2016

Immune and Endocrine Modulations Associated with Enhanced Plant-Based Protein Diets Within Freshwater Fish Species

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IMMUNE AND ENDOCRINE MODULATIONS ASSOCIATED WITH ENHANCED PLANT-BASED PROTEIN DIETS WITHIN FRESHWATER FISH SPECIES

BY

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A dissertation submitted in partial fulfillment of the requirements for the

Doctor of Philosophy

Major in Wildlife and Fisheries Sciences

South Dakota State University

2016

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This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy in Wildlife and Fisheries Sciences degree and is acceptable for meeting the dissertation requirements for the degree. Acceptance of this does not imply that the conclusions reached by the candidates are necessarily the conclusions of the major department.

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This dissertation is dedicated to my father, Douglas Bruce. Thank-you for always making science and discovery an integral part of growing up.

ACKNOWLEDGEMENTS

I would like to thank my wife, Larissa, for all of her assistance and support throughout my final chapter of graduate school. Thank-you for being there and tolerating all of my late hours spent at the lab during my trials. I would also like to thank my parents, Doug and Lyla, for all of your support and encouragement over the past four years. Further, I would like to thank the members of the Bruce clan and the Ellis family for all of their encouragement throughout my academic career.

Dr. Brown, thank-you for the research tools and academic training that you have provided for me throughout my Ph.D. program. I have obtained a profuse amount of invaluable experience and have become a better scientist because of your guidance. I am very grateful that you have provided me with the research opportunities and accompanied skill sets to effectively contribute to our rapidly expanding aquaculture industry. I would like to extend thanks to my committee members, Dr. William Gibbons, Dr. Alan Young, and Dr. Regg Neiger. I appreciate your support and innovative troubleshooting throughout my feeding trials and bench work. Special thanks is extended to Dr. Jeff Clapper for serving as my graduate faculty representative during my dissertation defense. Lastly, I would like to thank Dr. Ahmed Mustafa for your continued encouragement and the introduction to aquaculture physiology during my Master's degree.

This dissertation research and data collection would not have been possible without the support of Dr. Mike Barnes, Dustin Schulz, Scott Sindelar, Michael Grey, Brandon White, Tom Kasiga, Luke Fredrickson, Ashton Fey, Miran Hama-Sahl, Erinn Deslauriers, Hunter Brown, Caleb Green, Evan Koep, Spencer Gibbons, and Seema Das. Thank-you to Dr. Jason Bootsma, Dustin Gibbons, Michael Rinehart, Luke Leischner,

Nathan Morris, Jason Croat, and Dr. Liyan Chen for bioprocessing assistance. A great deal of thanks is extended to Dennis Harstad, Mark Leucke, Sue Lancaster, Dr. Steven Craig, Dr. Bill Harris, Dr. Marlies Betka, Dr. Daryl Basham, Dr. Ranjith Averineni, Dr. Joy Scaria, Dr. Hemachand Tummala, Jaimie Gibbons, and Dr. Jeffrey Clapper for project support.

These research endeavors would not have been possible without the funding support of Prairie AquaTech, the South Dakota Soybean Research and Promotion Council, the United Soybean Board, South Dakota Soybean Processors, the South Dakota Game, Fish and Parks, the National Science Foundation, the South Dakota Agricultural Experiment Station, the North Central Regional Sun Grant Center, and South Dakota State University, and the SDSU Department of Natural Resource Management.

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LIST OF ABBREVIATIONS

ACH: Alternative complement activity

AMP: Antimicrobial peptide

ANCOVA: Analysis of covariance

ANF: Antinutritional factor

ANOSIM: Analysis of similarities

ANOVA: Analysis of variance

AOAC: Association of Official Agricultural Chemists

APC: Antigen presenting cell

ASC: Antibody secreting cells

B: Bursa-derived

BCA: Bicinchoninic acid

BcR: B-cell receptor

Bp: Base pair

BSBM: Bioprocessed soybean meal

BSC: Biosafety cabinet

BSL: Biosecure laboratory

C: Celsius

Ca: Calcium

CCR: Chemokine receptor type

CD: Complementarity-determining region

cDNA: Complementary DNA

CFU: Colony forming units

Cl: Chloride

cm: centimeter

CRP: Complement reactive protein

CSPC: Commercial soy protein concentrate

Ct: Cycle threshold

DDGS: Distiller's dried grains with solubles

dH20: distilled water

dL: deciliter

DMB: Dry matter basis

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DTT: Dithiothreitol

EDTA: Ediaminetetraacetic acid

EGTA: Ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

EXOP: Exo-polysaccharide

F: Forward primer

FAO: Food and Agriculture Organization of the United Nations

FCR: Feed conversion ratio

FCS: Fetal calf serum

FITC: Fluorescein isothiocyanate

FM: Fishmeal

FMC: Fishmeal control

g: Grams

g: Gravity

ga: gauge

GALT: Gut-associated lymphoid tissues

GH: Growth hormone

GIT: Gastrointestinal tract

GnRH: Gonadotropin-releasing hormone

GVB: gelatin veronol buffer

h: Hours

HBSS: Hank's balanced salt solution

HPI: Hypothalamic-pituitary-interrenal

i.u.: International unit

IEL: Intraepitheial lymphocyte

IFN: Interferon

Ig: Immunoglobulin

IL: Interleukin

IP: Intraperitoneal

JAK-STAT: Janus kinase/signal transducers

K: Fulton's condition factor

K: Potassium

Kg: Kilogram

KOH: Potassium hydroxide

L-15: Leibovitz-15 medium

L: Liter

LPS: Lipopolysaccharide

LT: Lymphotoxin

M: Molar

MAC: Membrane attack complex

MALDI-TOF: Matrix-assisted laser desorption/ionization time of flight

MDFM: Marine-derived fishmeal

Mg: Magnesium

mg: Milligram

MHC: Major histocompatibility complex

mL: Milliliter

mm: Millimeter

mM: millimolar

MOS: Mannan oligosaccharides

mRNA: Messenger RNA

MS-222: Tricaine methanesulfonate

Na: Sodium

NBT: Nitroblue tetrazolium

NFE: Nitrogen free extract

ng: nanogram

NIH: National Institute of Health

NK: Natural killer cell

nm: Nanometer

nmol: nanomolar

OD: Optical density

OTU: Operational taxonomic unit

PAMP: Pathogen-associated molecular pattern

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PG: Peptidoglycan

PRR: Pattern recognition receptor

R: Reverse primer

RAG: Recombination activating gene

RAS: Recirculating aquaculture system

RBA: Respiratory burst activity

RBC: Red blood cell

RG: Relative Growth

RPM: Revolutions per minutes

rRNA: Ribosomal ribonucleic acid

RT-PCR: Real-time polymerase chain reaction

SAP: Serum amyloid protein

SBM: Soybean meal

SEM: Standard error of the mean

SGR: Specific growth rate

SIMPER: Similarity percentage analysis

SOCS: Suppressors of cytokine signaling

SPC: Soybean protein concentrate

SSI: Spleen somatic index

T: Thymus

TcR: T-cell receptor

TGF: Transforming growth factor

Th1: Type 1 T helper cell

Th2: Type 2 T helper cell

TLR: Toll-like receptor

TNF: Tumor necrosis factor

TYES: Tryptone yeast extract with salts

U: Units

WB: Wet basis

WSv: White spot syndrome virus

µL: Microliter

µm: Micrometer

ABSTRACT

IMMUNE AND ENDOCRINE MODULATIONS ASSOCIATED WITH ENHANCED PLANT-BASED PROTEIN DIETS WITHIN FRESHWATER FISH SPECIES

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2016

The incorporation of bioprocessed soybean meals (BSBMs) as a protein replacement for marine-derived fish meal (MDFM) has gained momentum with the potential for aquafeed cost reduction, improved growth performance, and reducing exploitation on wild fish stocks. Further, potential health benefits and optimized production performance resulting from bioprocessed feed ingredients have led to research on the nutraceutical benefits of plant-based ingredients[1]. A novel proprietary MBBM has been developed using a microbial conversion of carbohydrates into digestible protein and additional cellular components that have immunogenic potential. A series of five finfish feeding trials were conducted to investigate immunomodulation induced by the BSBM ingredients or dietary immunostimulants in yellow perch (*Perca flavescens*) and rainbow trout (*Oncorhynchus mykiss*), two domestically cultured species.

Nonspecific immune components were characterized by trials incorporating graded inclusion levels of polysaccharides and minerals. A 1-1.5% inclusion of a commercial exopolysaccharide (EXOP) with the BSBM provided enhancement to the phagocytic capacity and superoxide production of yellow perch head-kidney

[1.] Gatlin D.M., Barrows F.T., Brown P., Dabrowski K., Gaylord T.G., Hardy R.W., Herman E., Hu G., Krogdahl Å., Nelson R., Overturf K., Rust M., Sealey W., Skonberg D., Souza E.J., Stone D., Wilson R. & Wurtele E. (2007) Expanding the utilization of sustainable plant products in aquafeeds: A review. *Aquaculture Research* **38**, 551-579.

macrophages. Analysis of respiratory burst activity (RBA) after 46 days of treatment were found to be significantly different across dietary treatments ($P<0.01$). A 0.1% inclusion of commercial β-glucans ($P=0.03$) and 1% inclusion of EXOP ($P=0.03$) both produced greater macrophage superoxide production than the MDFM control diet. Phagocytosis rates were also found to be significantly different ($P=0.01$) following a 60-day feeding trial, with the 1% EXOP diet providing a greater phagocytic capacity than MDFM ($P=0.04$) and BSBM ($P<0.01$) control diets. In a follow-up innate immune study with mineral (zinc and selenium) supplementation, inclusion of zinc producing greater superoxide levels ($OD_{620}=0.548$) than the commercial exo-polysaccharide ($OD_{620}=0.326$) at short-term sampling periods. Study results indicate a variation in short-term innate immune responses as a result of feeding mineral (zinc and selenium) and polysaccharide (β-glucans and EXOP) supplemented bioprocessed soybean meals.

Rainbow trout were challenged with *Flavobacterium psychrophilum* (17830), a major salmonid pathogen in commercial aquaculture operations. At 10 days post-challenge, mortality rates were found to be lower in diets containing a base BSBM (47.91%), BSBM with 1% EXOP (49.55%), and BSBM with 0.1% β-glucans (40.47%), in comparison to the MDFM control diet (59.45%). At day 46, the macrophage RBA of the fish fed supplemented (EXOP and β-glucans) BSBM variants were greater than the FM control diet($P=0.009$ and $P=0.002$, respectively). Lysozyme levels were found to have significant differences among diets at day 46 ($P<0.001$) and day 60 ($P<0.001$), but no significant differences were found as lysozyme levels decreased across all treatments following the *F. psychrophilum* challenge ($P=0.695$).

Two 60-day feeding trials with juvenile rainbow trout (*Oncorhynchus mykiss*) were conducted to compare the effects of fishmeal (FMC), defatted soybean meal (SBM), bioprocessed soybean meal (BSBM), and commercial soy protein concentrate (CSPC) ingredients on intestinal histology, innate immunity, and microbiota profiles. Intestinal lysozyme content was found to be highest at day 60 in the BSBM treatment group ($P<0.01$). No significant differences across treatments were observed in histological profiles at day 0 ($P=0.75$), day 15 ($P=0.08$), and day 60 ($P=0.22$). 16S rRNA microbiota analyses from collected fecal material indicated differences in alpha diversity among dietary groups, as demonstrated by Chao-1 ($P<0.01$) and Shannon indices ($P=0.02$). The incorporation of processed soy-based proteins alters the microbial community composition within the rainbow trout gastrointestinal tract (GIT), and modulates lysozyme concentrations within the distal gastrointestinal tissues.

A 60-day feeding trial was conducted to evaluate physiological changes from acute stressors in rainbow trout (*Oncorhynchus mykiss*) fed soy-based diets. Experimental diets contained two BSBM levels or a CSPC while dietary controls included a FM reference and a SBM. On days 30 and 60, trout were subjected to 4 hours of acute stressors (low flow rate, decreased dissolved oxygen and handling stressors) or left unstressed as controls for respective diet treatments. Day 30 changes in macrophage RBA were significantly different among diet treatments in the stressed ($P=0.004$) groups and between the stressed and unstressed groups at Day 60 ($P=0.004$). Plasma cortisol results indicate that there were no dietary treatment differences but day 30 stressed (11.48 ± 1.51 ng mL^{-1}) and unstressed groups (0.47 ± 0.28 ng mL^{-1}) had a significant difference in group means ($P<0.001$). Day 60 plasma cortisol levels following a similar

trend, significance between stressed (7.22±1.48 ng mL^{-1}) and unstressed (0.36±0.12 ng mL^{-1}) groups ($P<0.001$). Continued assessment of novel plant-based ingredients to reduce stressors may benefit producers by allowing increased stocking densities and reducing losses due to fish transport, thus further contributing to the needs of a rapidly expanding domestic aquaculture market.

Small additions of immunostimulants and the incorporation of bioprocessed soybean meal appear to modulate the fish immune system, the distal intestine microbiota, and aspects of stress physiology. These findings corroborate current aquaculture immunology initiatives to create non-antibiotic approaches that enhance fish health and increase production stocks in the rapidly expanding aquaculture sector.

CHAPTER 1. A REVIEW OF IMMUNITY, STRESS PHYSIOLOGY AND GROWTH REGULATION IN FINFISH AQUACULTURE

Status of Domestic Aquaculture and Fish Health

Global aquaculture production has rapidly increased over the past few decades. Increasing world population size, standard of living, and globalization have created a large demand for high quality, protein-rich food. High quality protein acquired from fish and shellfish constitutes approximately 16 percent of the world's consumption of animal protein sources and may fuel the food production for future generations, along with the need for adequate sustainability of natural resources (Tidwell & Allen, 2001). This increased need from the growing world population has placed excessive demands on the wild-caught fishing industry and has driven growth of the aquaculture production sector. In 2012, global fisheries' production was estimated at 158 million tonnes, with 66.6 million tonnes of this amount credited to aquaculture production (FAO, 2014). Global food fish production attributed to aquaculture has grown at an average annual rate of 6.2% from 2000 to 2012, doubling annual production (32.4 million tonnes in 2000; 66.6 million tonnes in 2012) within this time interval (FAO, 2014). Furthermore, Asia accounts for nearly 90% of global aquaculture production, while North America, Japan, and Europe account for only one tenth of production, creating large import markets within those countries (Naylor et al., 2000).

The increasing culture of marine and freshwater teleost species requires a vast amount of feed ingredient – fishmeal. Fishmeal protein is comprised of a complete essential amino acid profile and has an appropriate allocation of minerals, fatty acids, and

vitamins (Jobling et al., 2001). The increase in fishmeal capture has been prominent worldwide, but Chile and Peru account for approximately 40% of the global fishmeal production, which is estimated at 5-7 tonnes annually (Naylor et al., 2009). Fishmeal is composed of a variety of smaller species and byproducts from fish prepared for human consumption. Menhaden, sardines, and anchovies are the primary constituents of whole-body fishmeal (Jobling et al., 2001). Large numbers of smaller composite fish may potentiate elevated fishmeal market prices and availability due to of overfishing, inclement weather, and migration in traditionally saturated area (Tidwell & Allen, 2001). With fishmeal, the dietary composition and protein ratios of product batches may fluctuate based on the supply species, and quality of fish. Thus, lipid and protein levels may require adjustment based on the requirements for the target fishes (Windsor, 2001).

Large-scale manufacturing of fishmeal for aquatic feeds can also create protein degradation and loss of amino acid availability due to temperatures and production processes (e.g. lipid extraction) in sizeable manufacturing operations (Pigott & Tucker, 2002). Furthermore, it is estimated that approximately 4kg of whole-body fishes is required to make 1kg of fishmeal product, rendering it an inefficient use of protein (Allan et al., 2000). The aquaculture industry utilizes approximately 50% of all fishmeal supplies, with swine and poultry retaining the remainder for feed applications (Alder et al., 2008). It has been suggested that the industry will increase use of seafood waste by-products to provide an additional 15-20% increase in bulk additive (Hardy, 2010). Once again, this may only be a short-term solution to the sustainability issue and may also elevate fishmeal prices from using more expensive seafood species or byproducts in contrast to the traditional smaller fishes. Increased demand or pricing based on fishmeal

availability could place major constraints on the worldwide development of the aquaculture industry.

The incorporation of plant-based protein as a sustainable fishmeal replacement has received considerable research interest in the past few decades. Fishmeal diets offer a nutrient-rich protein source, but the feedstuff is subject to high prices, diminishing supply, and an unsustainable demand (Adelizi et al., 1998). Consequently, over the past few decades researchers have sought to expand the utilization of plant-based protein products in an effort to maximize growth in comparison to fishmeal while reducing production costs. Fishmeal is the most expensive component of feeds and further, operating costs, of large-scale aquaculture; therefore, new cost-effective feeds are desirable for many producers (Naylor et al., 2000). Directly, there has been a marked increase in alternative protein inclusion levels following the deviation from complete fishmeal blends. It has been speculated that the protein constitution of fishmeal feeds will be reduced in salmon and trout diets as plant-protein alternatives become more prevalent within the production market (Tacon & Metian, 2008). Furthermore, fishmeal prices have soared upwards of $2,100 per tonne in 2013, an estimated 206% increase from January 2005 (FAO, 2014). Alternatively, commodity prices for alternative protein sources, such as soybeans, only slightly increased to approximately $400 per tonne (Olsen & Hasan, 2012). Thus, soybean meal offers an economic and advantageous addition to the aquaculture feedstock market. Defatted soybean meal offers a comprehensive amino acid profile, low lipid ratio, supply abundance, and inherent vitamins (Gatlin et al., 2007). The amino acid composition can be altered through nutrient supplementation to meet the feed requirements of many teleost species. Lysine,

methionine, cysteine, and threonine are essential amino acids that are often indicative of the nutritional value in soy protein concentrates (SPCs; Gatlin et al., 2007). Altering the amino acid composition may provide an enriched product that may equal or surpass fishmeal with additional amino acids. Soybean meals typically have a protein composition of 45%, which is adequate, but significantly lower than the fishmeal blends (Gatlin et al., 2007). Additionally, concentrates can be produced from the soybean matter through ethanol extraction to produce a higher protein blend that is more representative of fishmeal amino acid levels (Sales 2009). Research efforts have been increased to improve protein levels in soybean material to afford a more enhanced feedstuff for aquaculture production.

Despite the various offerings provided by soybean protein replacements, there are also antinutritional factors that must be addressed to improve digestibility and allow increased inclusion levels, particularly for piscivorous fishes. Lectins, saponins, protease inhibitors, and phytate are all naturally-occurring chemicals found in seed meals that need to be reduced or eliminated prior to use in feeds (Jobling et al., 2001). Lectins play a role in physiological carbohydrate regulation and must be heat-treated to increase digestibility in aquaculture feeds (Hendricks, 2002). Lectins have the capability of binding to organs and membranes that function in digestion, while inhibiting enzymatic release (Gatlin et al., 2007). The saponins are a class of triterpene glycosides that are present in soybean and may cause respiratory gill damage through leeching toxicity (Francis et al. 2001). Saponins can be extracted in a aqueous solution during meal processing, prior to use in aquaculture feeds. Digestibility of proteins from feed intake is drastically impacted by the presences of protease inhibitors. Protease inhibitors,

specifically trypsin inhibitor, allow for inappropriate peptide cleavage and reduced amino acid availability due to overproduction of digestive enzymes in the pancreas (Romarheim et al., 2005). The normal function of trypsin is to cleave peptides into their amino acid components for nutrient absorption and metabolism in the gastrointestinal tract. Cooking extrusion is used to degrade the protease inhibitors and permit increased digestibility of the soy protein. Excessive heating during extrusion may degrade beneficial protein levels, so an appropriate extrusion process must properly reduce inhibitor function without degrading protein. Lysine, for example, is readily affected by heating during extrusion (Hendricks, 2002). As a result, the sensitivity varies among fish species to the negative effects of protein inhibition. Phytate is responsible for a disruption of the phosphorus metabolism within soybean meals. Chelation is the mechanism of interruption for the phytate and it may also block the uptake of essential minerals such as calcium, magnesium, and zinc (Francis, 2001). Phytase inhibitors are additives in feed composition to curb the phytate activity and increase the bioavailability of organic phosphorous. Although antinutritional factors may hinder the optimal performance of soybean as a protein source in aquaculture feeds, there are many relatively simple and cost-effective processing measures to retain a high level of digestibility and protein content.

There have also been recent accounts of immunogenic properties of soybean meal in various aquaculture species and potentially providing an added health benefit. An investigation with Atlantic salmon (*Salmo salar*) and soybean meal dietary supplementation has yielded supporting evidence for immune enhancement (Bakke-McKellep et al., 2000). Following administration of soy diets, the salmon were found to

have increased concentrations of macrophages within the lamina propria of the distal intestine (Bakke-McKellep et al., 2000). There has been much debate whether soybean-based feeds have a beneficial or negative impact on the immune system, as some soybean-based diets may induce higher than normal immunoglobulin levels while limiting leukocyte recruitment numbers to respond to the pathogen. The predominant enteritis resulting from soybean feeds may create susceptibility for opportune infections, such as furunculosis caused by *Aeromonas salmonicida*. Alternatively, it has been observed that macrophage chemotaxis and antibody titers have been found to increase with the increasing levels of soybean-based protein within the aquaculture diets (Kiron, 2012). This increase may be attributed to less than adequate amino acid levels in the feeds: arginine, glutamine, and cysteine. Nucleotide supplementation has also been found to elicit an immunomodulation response on some teleost species. It has been found that appropriate nucleotide profiles are directly correlated with increased cytokine levels and gene expression of IL-1β and IgM throughout the kidneys (Low et al., 2003). Although fishmeal contains a relatively comprehensive amino acid profile, the enhanced soybean-based diets may offer larger inclusions of these immune-stimulating amino acids while promoting a more proficient digestibility for nutrient absorption and growth.

An optimized and efficient immune system is essential in order to combat many stressors and pathogens associated with aquaculture environments. Appropriately balanced and immune enhancing feeds may create a healthier population with sustained growth performance. Crowding, handling, and temperature are all production variables that may influence the growth capacity for tank systems while compromising the immune systems of the production species (Wedemeyer, 1997). Decreased dietary uptake has

been associated with stressful conditions in intensive aquaculture. When dietary protein is limited, the fish may allocate protein constituents to energy requirements in lieu of immune functions. This switch may result in a compromised immune response from the reduced production of cytokines and immunoglobulins, which are essential immune proteins (Fletcher, 1997). The prevention of stressful conditions and the application of advanced soy-based feeds that may alleviate depressed immune systems may have exponential economic implications for intensive aquaculture.

Cost-effective feeding applications are of upmost importance while attempting to obtain maximum growth and control pathogen interference in commercial aquaculture. The use of advanced plant-based feeds may offer an immune-enhancing approach at a lower price point in comparison to the use of traditional fishmeal feeds. Additionally, the oral administration for an immunoprophylactic feed eliminates the need for medication and costly vaccinations in response to depressed immune systems. The cost of prevention from a compound feed is far less expensive than future large-scale pathogen treatment. The relatively new application of soybean feedstuffs in aquaculture feeds creates many new opportunities for the blending of optimal immunoprophylactic feedstuffs. Next-generation dietary soybean blends are dependent upon immunostimulating properties and a reduction of the antinutritional factors. Future development and improved efficiency of immune-enhancing feedstuffs may promote more superior yields and sustainability from intensive aquaculture.

Teleost Innate Immunity

Maintenance of the immune system within teleosts is essential to their growth and physiological development. In aquaculture, broodstock health and expected yields hinges upon the fishes' abilities to protect themselves from external pathogenicity. To protect themselves from outside pathogens, teleosts have developed innate and marginally adaptive immune system components to combat many types of bacteria and viruses. The innate immune system directs nonspecific pathogen clearance through a variety of humoral and cellular responses (Magnadottir, 2006). The ability to eliminate foreign matter from an organism is dependent upon the capacity to recognize self versus non-self cells: a fundamental building block of immunology. Thus, the teleost immune system functions in a generalized capacity (the innate immune response) and a more complex defense (adaptive immunity) to satisfactorily cover a wide array of foreign materials.

The innate immune system includes many internal and external barriers, such as the skin and enclosed epithelial surfaces, to limit pathogen intrusion. The scales, mucous, and gastric environment all act in unison to effectively trap and expunge foreign microbes (Magnadottir, 2006). Further, cellular components exist within the plasma to combat organisms that have penetrated the epithelium. Phagocytic cells, including macrophages and granulocytes, monitor the interior spaces to aid in the inflammatory response and removal of bacterial components. Furthermore, nonspecific cytotoxic cells may direct microbial degradation via pattern recognition, and antigen-presenting cells (dendritic cells and macrophages) may transfer more specialized pathogenic components to the adaptive immune system (Magnadottir, 2006). The final component of the innate immune system involves the humoral response, where specialized plasma or epithelial components may diminish microbes through enzymatic degradation. These main

constituents work in concert to prevent infections and they may overlap in function to optimize protection for teleosts.

The teleost epithelial barrier is composed of an integument covered with protective scales for environmental protection. This barrier takes on an immunological role by separating the internal and external environments while secreting mucus and lysozyme to enhance the breakdown of microbes (Balasubramanian et al., 2012). Depending upon the epithelial composition, some fish may experience an increase of external disease resistance while showing no difference in disease susceptibility following bacterial infection (Ellis, 2001). In general, mucus is a secretion of viscous glycoproteins and mucin that acts to lubricate the outer anatomical structures that enhance osmoregulation, encourage microbial removal, improve swimming ability by reducing resistance, and assist with respiration (Balasubramanian et al., 2012).

The concentration and secretion rates of fish mucus will change with environmental conditions and secretion also be induced by stressful situations. Additionally, the concentration of mucus secretion varies greatly among different fish species and may vary based on the bacterial contact with the apical surface of the epithelium (Austin & McIntosh, 1988).

Mucus has been determined to contain lysozyme, anti-bacterial peptides, proteases, and lectins to protect against microbes (Austin & McIntosh, 1988). Antibacterial peptides, including pleurocidin, attack the cell wall A-layer components of invading bacteria and are predominately found on the skin and gastric linings of teleosts (Ellis, 2001). Lectins are proteins that bind to carbohydrates on bacteria to prevent further binding to the integument and target the cell for opsonization (Ewart, 2001).

Opsonization is the molecular process of marking a target cell with complement components in an effort to further promote stronger phagocytosis of the cellular component or activate lymphocytes for more enhanced or advanced pathogen clearance (Whyte, 2007). Various types of lectins, such as C-lectin and galactose-binding lectin, serve different immunological functions (macrophage activation, respiratory burst, etc.) and many are Ca^{+2} dependent in terms of their binding competencies (Ewart, 2001). Cathepsins and trypsin-like proteases function by lysing external bacteria cell walls (Ellis, 2001). These proteases target cell wall and membrane components in an effort to rupture the cell and inhibit infectivity. Lastly, lysozyme is a major enzyme that inhibits bacterial infection through the disruption of the 1,4 glycosidic bond in the bacterial cell wall (Paulsen et al., 2001). Lysozyme also inhibits gram-negative bacteria and can be found in the mucus, plasma serum, and major organs of teleosts. It is created by the macrophages and granulocytes; thus, it is found throughout vertebrate systems (Uribe et al., 2011). Lysozyme acts independently of the complement pathway for bacterial clearance and increases following bacterial attachment or the acute phase of the teleost stress response (Demers & Bayne, 1997). These epithelial barrier components are vital to the prevention of bacterial entry into fishes. If these factors fail to eliminate pathogen entry, the cellular components of the innate immune system are the next line of defense for non-specific removal.

Cellular components of the non-specific immune system provide protection from pathogens that have breached the primary barriers. The two classifications of phagocytic cells are granulocytes and monocytes/macrophages. Granulocytes include neutrophils, basophils, and eosinophils, and are characterized by the presence of major granule

components in the cytoplasm. The polymorphonuclear cells are important in phagocytosis of invasive bacteria and parasites. The head kidney (pronephros) is the major hematopoietic organ in teleosts and compares to the function of bone marrow in mammals (Crippen et al., 2001). It has been determined in a challenge study with *Vibrio anguillarim* that 85% of phagocytic cells in the pronephros are acidophilic granulocytes (Sepulcre et al., 2002). This composition may change depending on the immunological status of the fish. Macrophages are phagocytic cells that serve to remove bacteria via phagocytosis and act as antigen-presenting cells (APCs) for the production of antigens in the adaptive immune response. Macrophages utilize cell-mediated exocytosis and the production of super oxide radicals via respiratory burst to eliminate pathogens in the teleost system (Whyte, 2007). Pathogen-associated molecular patterns (PAMPs) are foreign configurations that signal an invading cell to system macrophages (Belosevic et al, 2009). The macrophages have pattern recognition receptors (PRRs) on their cell surfaces that allow for ligand binding to the specific PAMPs bound on incoming matter. It is this relationship that allows for the macrophages to function in a non-specific manner to remove a cell deemed as "non-self" to the system. Following the connection of the bacteria to the macrophage, the macrophage then primes the respiratory burst and the production of reactive superoxide intermediates (Belosevic et al., 2009). Various peroxides, peroxidases, nitric oxide derivatives, and chloramines are produced to attack the invading bacterium and slow the metabolism (Whyte, 2007). Various cytokines are employed to transmit directions to macrophages and direct effective phagocytosis. It has been hypothesized that IL-1β is responsible for phagocyte recruitment through the activation of chemoattractants and enhanced immune gene expression (Buonocore et al.,

2005). Additionally, macrophages also harness the ability to produce transferrin, a serum protein that is capable to binding excess iron from the plasma, in an effort to limit bacterial metabolism (Belosevic et al, 2009). In addition to the granulocytes and macrophages, natural cytotoxic cells (Natural Killer or NK cells) exist for the non-specific removal of parasites, tumors, and cells that have been virally infected (Whyte, 2007). These cells are classified as agranular in nature, do not require Major Histocompatibility Complex (MHC) class restrictions for removal, and are commonly found in the pronephros and spleen (Miller et al., 1998). NK cells also contain a C-type lectin receptor for binding to class I marked cells and contain the ability to both stimulate and inhibit cytokine production in regards to removing the targeted cell (Sato et al., 2003). Hence, NK cells may be thought of as quite independent of the other phagocytic cells and lymphocyte populations. There are various species of NK cells and their exact nonspecific defense mechanisms are still being explored. T-cytotoxic cells also exist to detect virally-infected cells, similar to the response of a human CD8+ T lymphocytes (Miller et al., 1998).

A major humoral defense mechanism in teleosts is the ability to remove bacteria and associated matter via the complement system. The complement system is partially conserved in many vertebrates and acts via the Classical, Alternate, and Lectin-mediated pathways (Magor & Magor, 2001). These pathways differ in their initiation molecules, but all facilitate the activities of Complement Component 3 (C3). The Classical and Lectin pathways use Complement Component 4 (C4), Complement Component 1 (C1), and Complement Component 2 (C2) analogues in conjunction with immunoglobulin fixation to direct the cascade while the Alternate pathway is stimulated from the binding

of C3 to the surfaces of pathogens (Boshra et al., 2006). In the Lectin pathway, Mannose-binding Lectin (MBL) acts as a C1 homologue to cleave C2 and C4 counterparts upon membrane attachment, and may operate in the absence of C1 (Magor & Magor, 2001). Furthermore, C3 is generated in the alternate pathway from the combination of Plasma factor B and C3(H2O) to continue the cycle (Zhu et al., 2013). Pentraxins, such as Complement Reactive Protein (CRP) and Serum Amyloid Protein (SAP) are involved with the lectin-binding mechanism for complement activation on the cell membranes (Magnadottir, 2006). In addition to the activation of teleost complement, CRP is also a powerful promoter of opsonization and can effectively aid in the removal of fungal invaders or parasites (Bayne & Gerwick, 2001). Within these pathways, the molecule C3 is the major component of the complement pathway and may cleave C3a or C3b subsets; C3a further promotes inflammatory responses while C3b may further facilitate phagocytosis with nearby cells or assist with cleaving C5 in an effort to further the cascade (Magor & Magor, 2001). C5a is again used as a chemoattractant for phagocytic cells while C5b is an initiating factor in the formation of the membrane attack complex (MAC; Ellis, 2001). C3 and C5 convertases are used to enhance the progression through the cascade and cleave new molecular subsets (Nakao et al., 2011). Ultimately, the complement exerts its' ability to extinguish pathogens by lysis with the MAC or targeting the cells for further removal via opsonization (Whyte, 2007). The complement pathway is an effective procedure for promptly facilitating a non-specific immune response in teleosts. The availability of specific teleost cytokines and enzymes orchestrate the progression of the innate immune system.

Teleost Adaptive Immunity

The conservation of many immunological components in the innate immune system of vertebrates also translates to the adaptive immune system of fish. The adaptive immune system provides a more specified approach for pathogen removal and is the secondary defense for the fish from the external environment. Adaptive immunity involves the incorporation of T (thymus) and B (bursa-derived) lymphocytes and the capability to conduct cell-mediated removals. More specifically, the specific response includes receptor interactions with T cell receptors, immunoglobulins, and antigen-presenting cells (Tort et al., 2003).

The teleost cell-mediated, cellular components serve the purpose of removing antigens based on their genetic code and recognition. B-cell lymphocytes are integral to the proper disposal of foreign matter and the humoral branch of cell-mediated removal. B-cells function by secreting immunoglobulins – antibodies – into the plasma to create an antigen-binding complex (Scapigliati et al., 2003). These secretory cells are analogues of the mammalian plasma cells and are known as antibody secreting cells (ASCs) or plasmablasts (Kaatari et al., 2009). The largest concentration of B-lymphocytes exists in the pronephros and, accordingly, this region is also rich in immunoglobulins (Danilova, 2002). The incorporation of surface-bound immunoglobulins on the membrane of the B-cell is also naturally occurring and allows for further identification and removal tactics. Following the creation of the antibody-antigen complex, the B-cell is capable of orchestrating phagocytic cells or the complement pathways for the removal of the target cell. This immunoglobulin, in conjunction with associated transmembrane proteins, constitutes the B-cell receptor complex (Scapigliati et al., 2003). The B-cell is

responsible for the appropriate allocation of immunoglobulins in the plasma and BcR complex and can induce immunoglobulin characterization and class-switching through V(D)J recombination events (Hansen et al., 2005). The class-switching and genetic rearrangement of the immunoglobulins allows for an extremely specific antibody-antigen interaction and the flexibility to sort for future antigen encounters.

Immunoglobulins contain heavy and light chain structures with respective constant and variable regions (Kaatari et al., 2009). In teleosts, IgM, IgD, and IgT (associated with the intestinal immunology) have been determined as antibody classes (Salinas et al., 2011). IgM is the most prevalent teleost immunoglobulin and takes on a tetrameric form as an antibody, allowing for eight separate antigen binding sites (separated by disulfide bonds) (Kaatari et al., 2009). IgM shares a similar multi-faceted formation with the mammalian form (pentameric), but in mammals, IgG is the most common immunoglobulin. IgM is commonly used in many immunoglobulin studies because it is most prevalent and the regions are well-mapped. IgM contains four constant regions of exons that constitute a conserved μ region (Savan & Sakai, 2009). It should also be noted that IgM may exist in a monomeric form, with the possibility of forming a tetrameric complex, within some teleost immune systems (Tort et al., 2003). IgD in teleosts is a relatively new discovery and involves alternate splicing at the c-δ region (Kaatari et al., 2009). IgD has been characterized and discovered in channel catfishes (*Ictalurus punctatus*) and it appears that IgD tends to be placed on B-cell membranes, perhaps adding excess to the BcR (Edholm et al., 2011). The IgT has yet to be fully characterized and it is quite dissimilar to both IgM and IgD. It is speculated that IgT contributes a protective role in mucosal and gastrointestinal regions, but it has also been

found to be present in zebrafish during early immunological development (Hikima et al., 2011).

T cells take on an intrinsic role in the cell-mediated adaptive immune system and direct immune function following the initial nonspecific defenses and provide an immunological response following antigen recognition with APCs. Following pattern recognition through the TCR, the T cell may then direct the foreign matter to removal via cytokine stimulation, phagocytosis or eliminate the cell with its own intrinsic function. Thus, the two main classes of T cells, T helper cells and cytotoxic T cells, elicit their cellular response depending on the MHC pairing and specific antigen-receptor interactions (Castro et al., 2011). The differentiation and regulation of T cells is dependent upon various influences from nearby immunological molecules. For instance, the helper T cells are regulated by antigen binding affinity, antigen concentrations, and signaling from APCs, while cytotoxic T cells are influenced by surrounding helper T cells and cytokine intensities (Gerard al., 2013). Additionally, the release of IL-2 has been found to act as a growth promoter for overall T cell development (Boschi et al., 2011). Following differentiation, helper T cells release IFN-γ to assist with combatting viral infections and lymphotoxin (LT) to assist with intracellular pathogens (Boschi et al., 2011). The classification of helper T cells can be further divided into classifications based on their functionality and targeted pathogens. The Th1 cell is responsible for providing cell-mediated clearance of microbes while the Th2 subset tends to target multifaceted pathogens, such as parasites (Yamaguchi et al., 2013).

Teleost T cells appear to be closely related to the mammalian subset and contain a complementarity-determining region 3 (CD3) binding domains within the TCR (Koppang

et al., 2010). The thymus and head kidney harbor T cell development and serve as a base for circulating T cells in the plasma. Koppang et al. (2010) have established the CD3 as an effective T cell marker to evaluate expression regions throughout fish species. Additionally, the TCRβ chain has been partially gene sequenced in rainbow trout and may act as another expression indicator for TCR mapping. Consequently, the TCRβ region has been found to undergo recombination and is immensely involved with viral recognition (Boudinot et al., 2001). It was found that large populations of T cells inhabit the thymus, while they are also present in kidney and splenic regions following development (Castro et al., 2011). In addition to T cell characterization, the antigen presentation of both major histocompatibility complex (MHC) class I and II has illustrated the directional activation of a T cell response. MHC class I interacts with the CD8+ cytotoxic T cells while MHC class II assists with complex formation in helper T cells (Castro et al., 2011).

Teleost Intestinal Immunity

The intestinal anatomy in teleosts provides antigen processing during digestion and propagates immune capacity. As external food sources enter the gastrointestinal tract (GIT) following feed uptake, the stomach and intestinal segments are essential to the removal of pathogenic material in an effort to conserve homeostasis. The intestinal region contains some structural similarity to the mammalian anatomical design and allows for an added degree of protection from ingested, pathogenic materials. According to Rombout et al. (2011), the teleost intestinal immune components contain both effector and inductor sites, which express a wide variety of functions. The effector sites contain

the gut-associated lymphoid tissues (GALT), such as Peyer's patches, while the inductor sites are constituted by the lamina propria and its intrinsic lymphocyte population. This combination of lymphocytes and immunoglobulins work in concert to provide an encompassing protection throughout the distal intestine. Additionally, there is a substantial population of T-lymphocytes (both $CD8^+$ and $CD4^+$), B-cells (as indicated by the presence of IgT), and cytokines to create a diversified immunological center (Martin et al., 2012). This dynamic assortment of immunological cells allows for enhanced antigenic uptake, swift clearance, and the ability to elicit a systemic immune response.

In the past, there has been a focus on the intestinal enteritis associated with the administration of soybean meal-based diets. The distal intestine is most susceptible to enteric inflammation as a result of the inclusive ANFs and alcohol soluble components of the feed ingredients (Romatheim et al., 2008). Enteropathy may be characterized by decreased vacuoles, increased leukocyte prevalence, decreased microvilli density, and a broadening of the lamina propria (Sahlmann et al., 2013). Furthermore, it has been found that a soybean meal inclusion of 5-10% may create some symptoms of enteritis in salmonids, necessitating the need for further feedstuff processing (Sahlmann et al., 2013).

Advanced bioprocessing and extrusion technology provides for the elimination of these enteritis-causing factors and has perpetuated the entry of soybean-based feeds into the aquaculture feedstuffs arena. Interestingly, Urn et al. (2008) found that a slight increase of 4°C in water temperature may also induce the rapid onset of enteritis following consumption of soybean diets. In another study with European sea bass (*Dicentrarchus labrax*), Torrecillas et al. (2013) found that treatment with mannan oligosaccharides increased leukocytes, goblet cells, and lymphocyte migration, thereby

indicating a pronounced immunological benefit from a natural feed additive. The additional bioprocessing of these feedstuffs is responsible for the deterioration of the ANFs and the enhanced digestibility of the protein components.

The teleost intestine contains many of the innate immune attributes found in other anatomical regions. The intestinal mucosa is a saturated substrate for leukocytes and they are harbored within layers of the lamina propria (Abelli et al., 1997). Within these strata are NK cells, lysozyme, and granulocyte proliferation; ultimately leading to increased production of superoxides and lysozyme (Nayak, 2010). In an additional role, the epithelial cells act as a secondary barrier to secure the passage while allowing for appropriate nutrient passages and a viable gut microflora to enhance digestion (Torrecillas et al., 2013). Macrophages in the distal portions of the intestine also aid as antigen-presenting cells, thereby allowing for antigens to interact with the adaptive immune system for recognition. In addition to the resident macrophages, other granulocytes are also found in the intestinal segments for innate clearance (Abelli et al., 1997). These innate populations are spread throughout the lamina propria and epithelial linings of the intestine, which allow for a close proximity to digested pathogens. Laboratory testing protocols seek to explore the competency of these innate immune cells using respiratory burst and phagocytosis assays, coupled with immunohistochemistry to evaluate distribution.

The adaptive immunity of the teleost gut has been well-studied; yet, many avenues remain to be explored. The lack of an organized GALT and varying pH changes through GIT passage allow for a diverse habitat of immune cells (Salinas et al., 2011). The intestinal regions have been found to have an inherent population of

immunoglobulins and isolated populations of both B and T cells, known and intra-epithelial lymphocytes (IELs; Fournier-Betz et al., 2000). Additionally, T cells have been found to be present in the midgut, harbored in the lamina propria. These T cells can be further divided into regulator and effector populations in order to examine the adaptive response (Picchietti et al., 2011). Immunoglobulins have been thought to be dispersed throughout various intestinal regions; yet, it has been concluded that there are some relatively localized segments that are rich in IgM. Following viral interference, mast-cell analogues have been determined to assist with inflammation and the indirect propagation of immunoglobulins (Dezfuli et al., 2008). Inami et al. (2009) found that there is a higher concentration of IgM-positive cells in the rectums of Atlantic cod, in comparison to the foregut and stomach regions. This finding coincides with the known adaptive immunocompetency of the distal intestine, and elaborates on the ability for immunoglobulins to aggregate in specified regions aside from traditional GALT. In terms of antigen presentation to the adaptive components, the distal to mid intestinal regions have been found to contain the greatest concentrations of MHCII molecules, and subsequently, lymphocyte pockets and dendritic cells (Fuglem et al., 2010).

Adaptive immune studies of the intestine have also created some speculation on additional immunoglobulins within the teleost system. Interleukins, IGFs, and COX genes have all been determined to play an essential role in cytokine signaling within intestinal layers (Nayak, 2010). As fish lack IgA, a penetrable immunoglobulin in mammalian systems, IgM is rapidly degraded within the harsh environment (Mulder et al., 2007). Thus, the need for proliferation and adequate cytokine signaling is integral to ensure a prominent internal defense. Ordas et al. (2012) has researched the CCR7

receptor for cytokine activation and regulation. It has been determined that this receptor recognized cytokine motifs and assists with the up-regulation of B and T cells within the teleost gut. A comprehensive determination of the role of intestinal cytokines will elucidate the mechanism of viral and parasitic invasions within fish species. Additionally, the role of IgT as a teleost immunoglobulin is also underway as a targeted research endeavor for gut immunophysiology and mucosal immunity. Cytokines associated with IgT regulation may enact undetermined components of mucosal immune response propagation, as the B-cell concentrations have been shown to be variable among teleost species (Salinas et al., 2011).

Recombination-activating gene 1 (RAG-1) is an essential component of the antigen-dependent reorganization of antibodies in the adaptive immune response of teleosts (Low et al., 2003). In the adaptive immune response, the antibody components rearrange their genetic structure to associate with their respective antigen components, thus allowing for efficient clearance. The RAG-1 is effective with developing B and T cell and its expression is aligned with the developmental immune stages in fishes (Mao et al., 2012). As such, RAG-1 expression may be used as a marker for studying the ontogeny of the teleost immune system through somatic growth stages. Interestingly, in a study of RAG-1 mutation in zebrafish, it was determined that reduced RAG-1 expression led to a down-regulation of adaptive immune cells (non-functional lymphocytes, NK cells, etc.) while neutrophil concentrations increased in a compensatory approach (Petrie-Hanson et al., 2009). There is only a single copy of RAG-1 available in the genome for expression; therefore, any genetic mutations during transcription of the gene may also result in immunodeficiency for the fish. RAG-1 is regulated by the nuclear protein ikaros

to properly direct appropriate antibody reception sites for the B cell population (Lin et al., 2008). Hence, the antibody determining regions of lymphocytes is organized in a complex manner and controlled by cytokines or genetic regulation at various organizational levels.

Future research holds much promise for a more complete understanding of this immune system segment. Many interesting findings have enveloped the more complex aspects of the adaptive immune processes with respect to systemic migration of immune cells and cytokines. A thorough understanding of the teleost intestinal immunology may provide some developmental comparisons for the mammalian immune system. Further investigation within this enteric system may also allow for enhanced vaccine delivery components, novel immunoprophylactic feedstuffs, and clarification of receptor interactions for defensive pathogen recognition.

Teleost Cytokines

Cytokines are modulators of immune function in both the innate and adaptive immune systems. These hormones exert their effect on a variety of immune cells and have the ability to spread quickly throughout areas of inflammation, bacterial invasion, and viral entry. The conservation of cytokines across vertebrates is clear and many teleost studies incorporate the monitoring of cytokine expression as a measure of immune competency (Secombes et al., 2009). Alternatively, the genetic homology between related cytokines is dissimilar within families, which may account for their ability to encompass a large scope of pathogens and their physiological complications (Huising et al., 2009). The major families of cytokines are the Interleukins (IL), Interferons (IFN),

Tumor Necrosis Factors (TNF), and Transforming Growth Factors (TGF; Secombes et al., 2009). Each of these subset members exerts their effect in a regulatory manner and may affect the progressive activity of both viral and bacterial removal. The TNF-A cytokine is one of the preliminary cytokines released following a malignant instigation and it contains the ability to induce future interleukins (Secombes et al., 2009). The TNF superfamily contains approximately 19 protein constituents and they are responsible for inducing immune cell relocation, apoptosis, and leukocyte differentiation (Garcia-Ayala et al., 2009). TNF-A has been found to initiate macrophages to the inflammatory site, command neutrophil migration, and heighten the respiratory burst potential (Kvamme, et al., 2013). The next phase in cytokine solicitation may be the interleukin modulation. The interleukins are also responsible for bathing the infectious areas with granulocytes and macrophages while further secreting additional cytokines to cleanse the region. There have been approximately 35 families of interleukins identified to date and many of these cytokines are secreted by T-helper cells, macrophages, monocytes, and endothelial borders (Secombes et al., 2011). The IL-B homologue has been detected in 13 teleost species and has been found to exhibit a conserved function of macrophage migration and T cell recruitment for advanced viral pathogens (Ellis, 2001). COX-2 is a pro-inflammatory gene that is also stimulated by IL-1B and aids in the expression of MHC class II macrophages (Secombes et al., 2009). Following further macrophage recruitment, the interleukins also contain the ability to activate cortisol release via the hypothalamic-pituitary-interrenal axis to alleviate symptoms associated with the infection (Kvamme et al., 2013). IL-B and IL-A are often first to be produced as a result of infection and may further propagate interleukin production for enhanced specificity to the

inflammation (Huising et al., 2004). For example, IL-6 may be recruited when encountering Gram-negative bacteria for the most efficient removal (Ellis 2001). Conversely, viral infections are often slated to IL-18 expression for the incorporation of IFNs and T-helper cell conscription (Huising et al., 2004). In addition, IL-4 is found to be an efficient promoter of Th2 differentiation while IL-2 stimulates the replication of T cells after an antigen has been presented to the adaptive system (Secombes et al., 2009).

IFNs are involved in multiple phases of the immune system, but mainly target the viral entry. There are two classes of interferons, Type I and Type II molecules, that produce various immunological responses (Robertson, 2006). Type I IFNs are produced by various immune cells following viral stimulation and are generally classified as being α or β (Robertson, 2006). Type II IFNs are elicited by T cells and NK cell types to enhance phagocytosis from macrophages and increased MHC I and II production (Secombes et al., 2009). This type is also referred to as the IFN-γ and this cytokine is summoned by increased concentrations of IL-12 and IL-18 (Robertson, 2006). In the case of many hormones, IFNs act by attaching to a specific receptor and they are mediated by regulatory factors, including the protein family Suppressors of Cytokine Activity (SOCS) (Zou & Secombes, 2001). The SOCS proteins target the Janus kinase/signal transducers and activators of transcription (JAK-STAT) pathway, which adjusts cellular functions based on cellular conditions and external stimuli (Shepherd et al., 2012). SOCS-1 and SOCS-3 act upon this JAK-STAT pathway to control various concentrations of system cytokines, thus eliminating inflammation and tissue damage that may occur with increased cytokine expression (Shepherd et al., 2012). As a result of this regulatory mechanism, it is clear that negative feedback for the SOCS proteins is crucial

to proper functioning of teleost immune systems, in addition to proper cytokine production. Aside from the negative inhibition, certain PAMPS, bacteria, viruses, and parasites have demonstrated the ability to induce SOCS expression (Wang et al., 2011). Furthermore, the teleost stress response, more specifically the release of cortisol, has been found to up-regulate the expression of the SOCS genes and create an inhibition of the regulatory cytokines (Philip et al., 2012). The discovery of SOCS proteins and their genetic constituents within yellow perch (*Perca flavescens*) was only recently discovered by Shepherd et al (2012). The expression mapping associated with these genes alludes to the complexity of cytokine regulation within teleost immune responses.

Cytokines derived from the TGF superfamily serve as modulators of lymphocyte availability and function while directing the activity of many other immune cells via chemotaxis (Garcia-Ayala et al., 2009). Differentiation of both lymphocytes and T cells are dictated by TGF-β levels and the molecule may play a role in reproduction or apoptosis of these immune cells (Zhu et al., 2013). By the same token, TGF-β ensures appropriate levels of the adaptive immune molecules to prevent excessive proliferation and adequate availability in the case of infectious onset. TGF-β has also been found to play an important role in intestinal immune regulation and may be essential to avoiding intestinal enteritis with many soybean-based products (Lilleeng et al., 2009). In short, the vast realm of cytokine signaling is imperative for the proper functioning of the teleost immune system. Further examination of cytokine expression provides researchers with relevant information towards immune system ontogeny, pathological signaling, and the administration of immunostimulants to provide benefits for fish.

Immunoprophylactics

The evaluation of immune-enhancing ingredients in aquaculture feeds has been a research subject of increasing interest in the past few decades. Many novel additives and administration methods have been studied to improve fish health. Fish that have enhanced and well-developed immune systems will provide increased aquaculture yields and profits. Traditionally, non-specific immune parameters have been studied in teleosts as injection was the most common administration route, and thus, response times were limited to shorter durations following treatment (Sakai, 1999). Injection may induce a fast-acting response, but may require repeat dosages that cause stress on the fish. The route of administration is an integral component to immunostimulant testing. Recently, immunostimulants have been incorporated into aquafeeds for oral delivery, therefore creating an extension of the immunoprophylactic efficiency (Gannam & Shrock, 1999). Oral (feed) administration alleviates the stressful injections, but an estimation and control of dosage is difficult, as the medication may have to be applied in an unpalatable vector. This delivery method is most practical for enhanced dietary blends that may serve as potent disease-resisting supplements. The combination of the immunostimulant offers the possibility of an easily digestible immunoprophylactic combined with a well-fitted dietary blend, thus ensuring appropriate dispensation and ensuring a regimen that may have long-term potential (dependent upon continual feeding schedules). Common immunostimulants are derivatives from bacteria and yeasts. Various microbial fermentation processes have been appearing in the plant-based feedstuffs market, thereby creating a need for immunoprophylactic testing to determine efficacy. The evaluation of

these enhanced dietary blends may clarify and identify important immune-enhancing properties that could alleviate expensive treatments or medications.

Previous studies with injected microbial components have elicited improvements to the innate immune system, including lysozyme production, increased complement activation, and have promoted macrophage function (Sakai, 1999). Components from bacterial cell walls and internal structures act by heightening availability of non-specific cellular components and raising antibody titers (Gannam & Shrock, 1999). Lipopolysaccharides, peptidoglycan components (PG), and *Mycobacterium* strains have all been previously implemented to heighten immune response in teleosts (Galeotti, 1998). The responses generated as a result of these treatments have been increased phagocytosis, respiratory burst, and T cell and B cell up-regulation. For example, Casadei et al. (2013) found that the feeding of PGs to rainbow trout significantly increased the amount of antimicrobial peptides (AMPs) over a 14-day time interval. The transcription of AMP mRNA is increased following bacterial injection, therefore creating an over-concentrated pool of available AMPs to combat infection and maintain an elevated presence (Smith & Fernandes, 2009). This ability to up-regulate AMP expression is linked to the ability to recognize PAMPs on the bacterial components. The PG in the Casadei (2013) study was isolated from bacterial sources, similar to the extraction of many immunostimulants from bacterial, plant, and viral sources. This extraction process from bacteria may be an efficient means of generating heightened immunity, but may prove too costly on a production scale. Consequently, many researchers have turned to more naturally abundant yeast or plant sources for their immunogenic properties.

Polysaccharide-derived immunostimulants have been favored for their ability to be transferred orally and their capacity to be incorporated directly into aquafeeds. β-glucans have emerged as a frontrunner in many teleost studies because of their availability for processing and capacity to enhance the immune system (Sakai, 1999). β-glucans are found in the cell walls of yeast and fungi with inherit β-1,3 and β-1,6 glycosidic linkages (Jorgensen & Robertson, 1995). Innate immune cells in teleosts have been found to have specific glucan receptors on the cell membranes that appear to stimulate phagocytic ability (Palic et al., 2006). Thus, a dosage–dependent concentration of β-glucans may be optimized for immune enhancement. Furthermore, similar glucan forms are also found in the fibrous components of oats and barley, which are commonly used in agricultural feeds (Galeotti, 1998). The inclusion of these feed ingredients in conjunction with bioprocessing may create new variations of immunoprophylactic feeds. In a capacity similar to bacterial injection, the β-glucan is responsible for increasing macrophage activity, respiratory burst, and cytokine production (Misra et al., 2006). Therefore, it appears that many of the innate immune parameters are modulated with the specialized feeds. Although many of the β-glucan teleost studies target innate immune responses, some adaptive immune characteristics have also warranted investigation.

Cell-mediated immunity components and cytokine dynamics may change depending on administration route, concentration of glucan administered, and teleost species. For example, Chansue et al. found a slightly increased array of cytokine expressions in Nile tilapia following oral dosages of β –glucans (Misra et al., 2006). IL-β and TNF were evaluated using anti-human cytokine protein components and ELISA; both hormones are known for their interactions with T and B lymphocytes. Conversely,

Rodriguez et al. determined that injection with β-glucans in zebrafish resulted in much more elevated IL- β and TNF levels (Rodriguez et al., 2009). These authors suggest that the immune induction of β-glucans may up-regulate transcription in particular regions, creating the promotion of possible cytokine variations (e.g. TNF-α1 and TNF-α2) with different effector functions. This variation may be further examined in a variety of fish species. Adjusting the glucan concentration and induction schedule may produce optimized applications for individual species.

Immunostimulant testing most certainly warrants further investigation and quantification for exact efficacies. As feed manufacturers move to bioprocessed plant-based feed ingredients, immunostimulant testing may reveal hidden health benefits that may improve aquaculture production.

Soybean Aquaculture Diets

The application of soybean-based ingredients in aquafeeds has become of recent interest, largely in response to rising fishmeal prices. Among seed meals, soybean meal offers a good protein content and a comprehensive array of essential amino acids. Soybean protein offers a comparable amino acid profile to fishmeal, but often require supplementation. Methionine and lysine are traditionally deficient and require additions into the diet. Although soybean diets appear to fill the gap in terms of fishmeal replacement, the plant material may require specialized processing to increase protein concentration and improve digestibility. Extrusion technology, bioprocessing, and genetic manipulation are all important aspects of creating an optimal soybean-based aquafeed. Extrusion forces the feed ingredients through high pressure and heat to

gelatinize and form pellets. Through this extrusion process, the diet will become more digestible, and palatable for the fish species because of specific control over pellet properties. Despite these benefits, high temperatures may also cause protein degradation and alter the protein components. Bioprocessing offers an alternative methodology by allowing protein breakdown into smaller peptides that may be more resistant to protein degradation via extrusion temperatures. Thus, new bioprocessing developments may result in innovative feedstuff with improved proximate compositions. Lastly, the genetic manipulation of both fish strains and soybeans has been discussed as a technology for improving plant-based aquafeeds (Gatlin et al., 2007). Specific strains of hatchery fish may be bred to tolerate soy-based feeds and perform at elevated soy inclusions. Alternately, soybeans may be genetically manipulated to produce superior nutritional profiles while demonstrating smaller amounts of antinutritional factors (ANFs).

Salmon and trout diets have traditionally contained up to 20 percent defatted soybean meal, with successful growth performance (Hardy, 2003). Various researchers have found that raising this level of inclusion may result in reduced growth as a result of lowered palatability. Fishmeal has a common protein content of 65-70 percent (dry matter basis) while additional processing and modification is required to create soybean-based feedstuffs of comparable protein (Hardy, 2010). A streamlined manufacturing process of soybeans is essential to profitable and marked performance. Appropriate extrusion or bioprocessing steps will create a feedstuff at a very reasonable price, relative to the rising global fishmeal prices. Thus, obtaining appropriate inclusion rates while maintaining a well-supported soybean dietary blend may provide extensive evidence for the sustainable transition from fishmeal.

Inclusion rates on growth performance and palatability have often been the focus of fishmeal replacement studies. In a recent Atlantic cod study, it was determined that a fifty percent fishmeal replacement was appropriate for the introduction of soybean concentrates (Colburn et al., 2012). Colburn et al. (2012) discovered only slowed growth in cod with full fishmeal protein replacement and did not encounter any signs of gastric enteritis. Kasper et al. (2007) also found that a fifty percent fishmeal replacement with soybean meal was recommended for the rearing of yellow perch using extruded and un-extruded soybean meals. Conversely, it was determined that fishmeal replacement of 20-30 percent soybean meal (SBM) in yellowtail provided better fillet yield and a better feed conversion ratio (FCR) than 40-50 percent soybean meal replacement (Tomas et al., 2005). As such, it is apparent that the species physiology in response to soybean concentrates must also be considered, as each soybean diet must be formulated to meet the nutrient requirements of each species. Nutrient requirements have been determined in many species, facilitating diet formulation to allow an appropriate inclusion rate of soybean products (Sales, 2009).

In addition to bioprocessing, novel additives have also been incorporated into soybean aquaculture diets. The addition of nucleotides has been of recent interest and has been implemented to assist with enhanced growth in soybean diets. Nucleotide addition refers to the process of adding genetic constituents in the effort to provide more strata for somatic growth and development. More specifically, nucleotide addition has been found to enhance cytokine expression levels, assist with development of the GI tract, and promote an increase in fillet mass within some species (Li & Gatlin, 2006).

These benefits may be of importance in soybean diets as they can compensate for some nutritional shortcomings while creating a possible immunoprophylactic effect.

Stress Response in Finfish

The teleost stress response is of utmost importance in the aquaculture industry. Increased interactions with humans in an unnatural environment may elicit various stages of stress and ultimately result in disease and poor yields. Common aquaculture stressors such as crowding, hypoxia, temperature fluctuation, and handling may all act as primary stressors. The stress response in fish may be divided into three main stages: primary, secondary and tertiary (Barton, 1997). The primary stage begins with an immediate response to acute stressors through adrenergic release and subsequent cortisol level fluctuation. The secondary response then coincides with metabolic needs and shifts the storage of glycogen to immediate usage as glucose, while creating various metabolites in the process. Finally, in the tertiary stage, the fish is subject to drastic changes in growth, reproduction, and an inhibition to the immune system. Upon initiation of a stress response, treatment and medication may become costly to inhibit physiological changes in the stock. Thus, the elimination of stressors is crucial to increase both fish health and profit margins.

The adrenergic stress response is the first to exhibit physiological effects following acute stressors. Epinephrine and norepinephrine are the primary catecholamines responsible for minimizing the physiological effects of stress, and elicit a 'flight or fight' regime for immediate survival (Perry & Bernier, 1999). Chromaffin cells of the anterior kidney are responsible for catecholamine release following sympathetic

stimulation (Perry & Bernier, 1999). The release of these hormones will provide fish with an added energy release to assist with maintaining homeostasis or removing itself from the stressor. This primary response is also responsible for invoking the immediate enhancement of innate immune system components (Easy & Ross, 2010). The activation of macrophages, increased lysozyme, and complement activation are all up-scaled to avoid any pathogenic entry during this unbalanced state (Demers & Bayne, 1997).

Lastly, the influx of cortisol is an integral component and biomarker for the stressed state. Cortisol functions by regulating glucose levels, mediating metabolic elements, and interacting with immune cells (Sumpter, 1997). Cortisol levels increase with stress and release is dependent upon the HPI-axis, similar to mammalian endocrinology. In terms of immunological influences, cortisol release appears to display a wide array of physiological functions. Cortisol serves an immune modulation by interacting with leukocytes and macrophages, and inducing cytokine release to immune signaling (Espelid et al., 1996). Conversely, cortisol also has the ability to suppress the adaptive and innate immune cells and immunoglobulins, dependent upon cortisol concentrations (Chen et al., 2002). As such, the monitoring of cortisol is helpful for deciphering immune characteristics and the influence of the corticosteroid on the developing immune responses.

Glucose and metabolic change are analogous with secondary stress physiology. Following the primary response, it is common for the feeding response to be inhibited with the adrenergic surge (Schreck et al., 1997). In an effort to complete the nutrient demands and metabolic needs of the fish, glycogen will be converted to raise blood glucose levels under the control of cortisol (Pottinger & Pickering, 1997). This

additional glucose will translate into available energy to alleviate the stressor. The elevation of blood glucose is an important test parameter for evaluating the secondary response. Additionally, metabolism is affected with the onset of the secondary stage. The ionic composition of the plasma is drastically changed as sodium influxes arise and the water is passed through the organism with little gain (McDonald & Milligan, 1997). This change creates issues for proper circulation and osmoregulation for the fish. Vasodilation in the gills may create respiratory deficiencies and increased sensitivity to water salinities or contaminants (Harper & Wolf, 2009). This inadequate gill function creates more strain on the fish heart rate and blood circulation, once again stimulating more primary stress. These secondary responses may be closely tied to acute stressors, but prolonged secondary outcomes often link to more detrimental tertiary implications.

The tertiary stress response is rather encompassing and influences reproduction, growth, development, and immune response. This phase is often a result of chronic exposures to stressors and has many negative outcomes for unprepared aquaculture operations. As energy and resources are diverted to the basic physiological needs for survival, many of the complex metabolic processes are compromised, which are often less favorable to growth and development. Reproduction is a process that is retarded during this phase. The escalating hormone cortisol has prolonged effects on the Gonadotropin-releasing hormone (GnRH) pathway and homeostatic levels of testosterone and estrogen (Pankhurst & Van Der Kraak, 1997). Thus, oocyte maturation and gametogenesis may be delayed or may not be within the realm of capability (Harper & Wolf, 2009). This may impact broodstock supply and create future spawns that are below expectations in size. In reference to growth following stress, teleosts are once

again drained of any additional energy expenditures. Stress has been linked to increases in GH levels and the provision of feeding satiation during stressful events, in an aim to conserve unneeded energy on feeding (Pankhurst & Van Der Kraak, 1997). Additionally, it has been speculated that stress may also influence thyroid secretions in an effort to curb metabolic demands (Pankhurst & Van Der Kraak, 1997). By focusing efforts away from somatic growth, the fish is able to redirect nutrient usage and energy to help curb the effects of stress.

The immune component of tertiary stress response is well–documented, but new adoptions to the literature are consistently being updated. Once again, this response is dependent upon effects from adrenergic release and cortisol stimulation over a long period of time.

Research Questions and Study Significance

The development of enhanced soybean aquacultural feeds provides a multitude of avenues for experimental research, as fishmeal has been the traditional dietary protein source.

The experimental series in this dissertation investigated the following main questions:

i) To what extent does enhanced soybean concentrate, as a protein supplement, modulate and improve the innate and adaptive immune systems of commonly cultured species (*Perca flavescens* and *Oncorhynchus mykiss*)?

ii) Do representative characteristics of the teleost stress response become enhanced following the administration of an enhanced soybean-based diet and introduction to acute stressors?

iii) To what extent do bioprocessed soy ingredients enhance the immune response to *F. psychrophilum*, a major bacterial pathogen in salmonid aquaculture?

iv) Are there major changes to gut morphology, immunity, and microbiota following the inclusion of bioprocessed soy ingredients in aquaculture feeds?

The immunological study of high quality soybean products in aquaculture feeds provides an overview and insight for feed formulators and manufacturers, as well as aquaculture producers. A sustainable and highly digestible protein feed ingredient with inherit immunoprophylactic properties may produce an added benefit and value in the search for viable fishmeal replacements. The incorporation of these ingredients into aquafeeds may produce profitable growth and yields for the modern aquaculturist while reducing or eliminating the expensive costs of antimicrobial and antiviral medications. Traditional medications or treatments can become costly when applied to large production fish stocks and may reduce income from operations.

The transition to high profile soybean products would create a more sustainable alternative to fishmeal-based feeds and increase the efficiency of many aquaculture operations. The targeted beneficiaries of these studies would most likely be fish producers, government hatcheries, and soybean farmers. Fish producers would benefit from the increased performance, healthier stock, and lowered medication costs. State hatcheries may observe increased stocking growths and require fewer funds allocated to treatment needs. The soybean producers may also have value-added benefits to their yields. A product that has an innate immunological effect in fish species may provide similar results in cattle, swine, and poultry feeds, thus creating a large market for soybean products in the agricultural feed industry. The potential health benefits associated with

these bioprocessed ingredients would allow for the global fish market industry to steer away from wild catches and stimulate local economies with both increased soybean harvesting and aquaculture yields.

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CHAPTER 2. IMMUNOMODULATION IN YELLOW PERCH *Perca flavescens* FED BIOPROCESSED SOY-BASED DIETS WITH INCREMENTAL INCLUSION LEVELS OF COMMERCIAL EXOPOLYSACCHARIDE

Abstract

Plant-based feedstuffs used as fish meal (FM) protein replacements have the potential for reducing feed costs and providing comparable performance in cultured fishes. Further, bioprocessing has become a major manufacturing component of plant-based ingredient production and these microbial conversion processes have provided enhanced protein content, elevated protein digestibility rates, and decreased antinutritional factors (ANFs). Also, immunostimulatory or antigenic coproducts may be present in the bioprocessed ingredients following these microbial modifications. A 60-day feeding trial using bioprocessed soybean (BSBM) diets supplemented with a microbial exopolysaccharide was performed with juvenile yellow perch (*Perca flavescens*). The study was conducted in a recirculating aquaculture system and BSBM diets containing 0.5, 1.0, 1.5, 3.0 or 6.0% of the exopolysaccharide were fed twice daily. FM and BSBM diets were included as dietary controls. Fish were sampled on Day 0, 14, 28, 42, and 60 to collect sera, head kidney macrophages, and splenic tissue. Pronephros-derived macrophages were cultured and assessed for respiratory burst activity (RBA) and phagocytic capacity using flow cytometry. Serum samples were assessed for alternative complement activity, lysozyme content, total protein, and total immunoglobulin levels. Day 60 RBAs indicated a significance difference between dietary treatments (P=0.02), with the 1.5% exo-polysaccharide diet producing the greatest RBA (OD_{620}=0.730). Day

60 macrophage phagocytosis were found to be significantly different (P=0.01), with the 1.0% inclusion having a greater phagocytic capacity than the FM reference (P=0.04), the BSBM reference (P<0.01), and the 0.5% Diet (P=0.01). Thus, targeted exopolysaccharide concentrations in bioprocessed plant-based feedstuff may provide an enhanced immune response for aquaculture species.

Introduction

Aquaculture is one of the fastest growing segments of agriculture, rivaling production from wild capture fisheries (Naylor et al., 2005). Fish meal (FM) has been a traditional protein source in aquaculture diets but market fluctuations, overfishing, and increased demand have all created an urgency to develop novel aquafeed ingredients (Olsen et al. 2012). Alternative feedstuffs, such as defatted soybean meals (SBM), fermented SBM (FSBM) and soybean concentrate (SPC), currently provide partial FM replacement in feeds. These ingredients provide a non-fishmeal protein source at a lower cost, offer a somewhat analogous amino acid profile, and allow for growth rates that are comparable to the marine-derived FM (Zhou et al. 2005, Heikkinen et al. 2006). Further, processing technologies applied to plant-based ingredients can improve protein and amino acid content and digestibility, and reduce or eliminate antinutritional factors (ANFs) that, in particular SBM, negatively impact growth and development (Kim et al. 2010).

Parallel to the increased use of plant-based nutrients, effective disease prevention and treatment regimens are essential to maximize yields, economic gains and increase trade of aquaculture products (Bondad-Reantaso et al. 2005, Fofano et al. 2012).

Potential immune system enhancements resulting from both bioprocessed plant-based proteins and polysaccharides in aquafeeds may increase pathogen clearance, decrease stress responses, and increase innate and adaptive immune parameters (Zahran et al. 2014, Schmitt et al. 2013, Sitja-Bobadilla et al. 2005). Microbially derived polysaccharide supplements, such as β-glucan and mannan oligosaccharides (MOS) have been found to impact fish immunity, disease resistance, and gut health (Fuchs et al. 2015). Furthermore, these levels of immunostimulants may be a direct result of inherent single-cell microbes used in feed ingredient bioprocessing, as some microbes have the ability to generate polysaccharides beneficial to animal health (Muramatsu et al. 2012, Jung et al. 2007). Thus, the objective of this study was to investigate graded levels of a commercial exopolysaccharide (EXOP) to characterize immunomodulation responses in yellow perch.

Materials and Methods

Each fully randomized experiment diet included purified EXOP (Shandong Freda Biotechnology Co., Ltd, Jinan, China) at increasing inclusion levels 0, 0.5, 1, 1.5, 3, and 6% of the dry diet. The 6 EXOP diets also contained BSBM, which replaced 50% of the dietary FM ingredient. Test diets were also compared against a FM reference diet of similar composition, with 4 replications of each treatment. Immune responses were assessed at day 14, 28, 42, and 60, with the day 60 fish sampled from a concurrent growth trial using the same experimental diets. Fish were randomly collected from each treatment at the designated time intervals for head kidney, spleen, and serum samples.

Diet formulations were based on published (Hart et al., 2010) and unpublished internal data for the nutritional requirements of yellow perch (Table 1). EXOP content of BSBM was low (0.01% dmb) due to deliberate manipulation of fermentation conditions and therefore was not considered as EXOP additions. Analyses of primary protein sources were completed by certified private laboratories for crude protein (AOAC 2006, method 990.03), crude fat (AOAC 2006, method 990.03), crude fiber (AOAC 2006, method 978.10), moisture (AOAC 2006, method 934.01), and ash (AOAC, method 942.05) and amino acids (AOAC 2006, method 982.30 E(a,b,c); Table 2).

The 60-day immunological trial was performed in a recirculating aquaculture system (RAS). Juvenile yellow perch (58.3±1.8g) were stocked (17 fish/tank) in 28, 110L tanks interconnected to a RAS and fed a FM reference diet, a BSBM diet with 50% FM replacement, or BSBM treatment diets containing graded levels (0.5-6%) of exopolysaccharide (Freda Biotechnology Co; Shandong China) (Table 1). Experimental diets were formulated to contain 42% protein and 9% lipid. The following calculations were made for splenosomatic index (SSI) and Fulton's condition factor (K-value):

$$\text{SSI}=\frac{\text{fresh spleen weight (g)}}{\text{whole fish weight (g wet)}}\times 100$$

$$\text{K} = \frac{\text{final weight (g wet)}}{\text{total length (mm)}^{3}} \times 100{,}000$$

Macrophages were isolated using methods by Secombes et al. (1990), with the temperature modifications for yellow perch. Head kidney isolates were removed aseptically and stored on ice in 2mL of Leibovitz-15 (L-15) medium containing 2% fetal calf serum (FCS) (Sigma-Aldrich, St. Louis, MI), 100 i.u. penicillin-streptomycin mL^{-1} (Sigma-Aldrich), and 10 units of heparin mL^{-1} (Fisher Scientific, Waltham, MA).

Pronephros samples were processed within 24h of extraction and the tissues were passed through 100µm mesh in 2mL of the modified L-15 medium. The samples were then placed on a 34%/51% Percoll (Fisher Scientific, Waltham, MA) gradient and centrifuged at 500 x g for 30min. Cells were collected at the density interface and added to cold, modified L-15 medium. The cell solutions were then centrifuged at 1000 x g for 10 min and the pellet was re-suspended in L-15 supplied with 0.1% FCS. Samples were adjusted to $2x10^7$ cells mL^{-1} using a hemocytometer, verified for viability using trypan blue, and $2x10^6$ cells were seeded in each well of a 96-well tissue culture plate. Cells were allowed to adhere for 2h at 23°C before being washed with fresh L-15 (non-supplemented). Adherent cells were incubated overnight in L-15 then supplemented with 5% FCS prior to the assays.

Blood was collected using a caudal vein sever, pooled for three fish per tank, and then deposited into sterile 2mL centrifuge tubes. The blood samples were allowed to clot at 4°C overnight and then centrifuged at 1000 x g for 10 minutes to isolate serum. Isolated sera was frozen at -80°C, pending assays.

Macrophage respiratory burst activity (RBA), derived from the pronephros was determined using spectrophotometric analysis of Nitroblue tetrazolium (NBT) reduction via superoxide production. For the RBA reagent preparation, NBT was added at a concentration of 1mg mL^{-1} and 1µg mL^{-1} phorbyl 12-myristate 13-acetate was added to L-15 medium. A 100µL aliquot of the RBA reagent was added to the wells and allowed to react for 30min. The wells were then emptied, the cells fixed with methanol, and the wells were washed twice with a 70% methanol rinse. A 120µL aliquot of 2M potassium hydroxide (KOH) and 140µL of dimethyl sulfoxide (DMSO) were added to each well

and the plates were agitated and read in a microplate reader (BioTek Corp., Winooski, VT) at 620nm, using KOH/DMSO wells as blanks. OD_{620} readings were calibrated to blanks and were compared to the respective RBA.

Flow cytometry was used to analyze phagocytic capacities of cultured macrophages fed fluorescent beads. Briefly, cell culture plates were washed with 100ul of L-15 media. 100 µL of L-15 (5%FBS) and 9µL of 0.92µm fluorescent beads (Fisher Scientific, Waltham, MA), approximately 1:10, were added to the treatment wells. Plates were incubated at 20°C for 1h and wells were then washed 2-3x with 1xPBS (pH 7.2). A 50µL aliquot of Accutase detachment media (MP Biomedical, San Diego, CA) was added and incubated at 20°C for 10-15 minutes. Cells were washed free from the wells with pipetting, transferred to a 96-well, round-bottom plate and centrifuged at 800 x g for 8-10 minutes to pelletize the cells. The pellet was re-suspended in cold paraformaldehyde (2%) and cells were quantified using a FACSCalibur (BD Dickinson, San Jose, CA).

Serum lysozyme was measured using a turbidimetric assay with lyophilized *Micrococcus lysodeikticus,* to determine inherent concentrations (Kim et al. 2006). Briefly, 100 ml of serially diluted serum in 0.05M sodium phosphate buffer (pH 6.2) in 96-well plates. 100 ml of 0.4 mg ml^{-1} suspension of *M. lysodeikticus* (Worthington Biochemical) in sodium phosphate buffer (pH 6.2) and the plates were agitated for 20s. The optical density (O.D.) was measured at 570 nm at 0 and 15 min at 20°C. Sodium phosphate buffer was used as a plate blank and replaced serum as a negative control. A unit of lysozyme activity was defined as the amount of serum causing a decrease in the absorbance of $0.001min^{-1}$.

Total immunoglobulin (Ig) was determined using previous methodology (Siwicki et al 1993). Briefly, serum dilutions (1:32 and 1:64) were made in a 96-well plate with 0.85% saline solution and bicinchoninic acid (BCA) protein assays (Pierce Biotechnology) were used to read total protein in the serum. 100µl of serum was added to 100µl of a 12% polyethylene glycol, incubated for 2h with shaking, and centrifuged at 1000 x g to collect the supernatant. The supernatant was then processed using the BCA protein kit and the difference in absorbance calculated from the total protein calculation to derive total immunoglobulin content.

Alternative complement activity (ACH50) was determined using the hemolysis of sheep red blood cells (RBCs) according to previously described and published methods (Pionnier et al. 2013). Sheep RBCs (10% packed volume in Alsever's solution; Lampire Biological, Pipersville, PA) were washed 3 times with 0.85% saline and suspended in gelatin veronol buffer (GVB) (10mM barbital, 145 mM NaCl, 0.5 mM NaCl, , 10 mM $MgCl_2$, 0.15 mM $CaCl_2$, 1% gelatin; Boston Bioproducts, Boston, MA). The cell solution was adjusted with a hemocytometer to a final concentration of $8x10^6$ RBC mL^{-1}. In a 96-well, round-bottom plate, yellow perch sera was serially diluted in GVB (from 0.5% to 7% concentrations). Positive controls were used for each sample to represent 100% hemolysis (100 µL of diluted perch serum, 100 µL dH_2O and 100 µL RBCs). For the previously prepared sera dilutions, duplicate sample tests (100 µL of diluted perch serum, 100 µL GVB with 10 mM EGTA, and 100 µL RBCs) were incubated for 1h at 20°C, alongside the controls. To stop the reaction, the samples were then centrifuged at 400 x g for 8min at 4°C to stop the hemolysis. 200 µL of the supernatant was relocated to a 96-well, flat-bottom plate density (OD) and read at 410nm (BioTek EPOCH, Winoota

VT). For each serum dilution, the hemolytic rate was calculated by dividing the OD value for the sample test by the OD value for the positive control. The ACH50 was calculated in ACH50 units/mL, and correspond to the serum dilution yielding 50% RBC hemolysis.

Analysis of variance (ANOVA) was used to compare immune metrics, while Tukey's HSD was used for multiple comparisons, when applicable. Nonparametric data were analyzed with the Kruskal-Wallis one-way analysis by rank and Mann-Whitney U tests for comparisons. Results from statistical analyses were deemed significant at α=0.05.

Results

The day 0 baseline RBA OD_{620} ranged from 0.270 to 0.340 and was considered as a baseline reading (*P*=0.84, Figure 1). There were slight increases in RBA at the day 14 sampling, but results were not significant (P=0.16). The BSBM control (OD620=0.50), 0% inclusion (OD_{620}=0.50), and the 6% inclusion (OD620=0.47) appeared to be elevated, while the FM reference remained lower (OD620=0.37). Day 28 results also did not yield a difference in RBA (*P*=0.26), and all dietary treatments appeared to be lower than the FM reference (OD_{620}=0.04) and the 6% inclusion (OD_{620}=0.37). Day 42 RBA was different among dietary treatments (*P*=0.04) (Figure 1). The FM reference elicited the highest RBA (OD_{620}=0.78) and was greater than the BSBM Control (*P*=0.04), the 0.5% inclusion (*P*=0.01), the 1.5% inclusion (*P*=0.03), and the 6% inclusion (*P*=0.01). The 1% inclusion was found to have the greatest RBA of the EXOP treatments (OD_{620}=0.74) and was greater than the 0.5% inclusion (*P*=0.04) and the 6% inclusion (*P*=0.04). Thus, it appeared that at day 42, the 0.5% inclusion created intracellular superoxide levels that were comparable to FM (*P*=0.55) and the 3%

inclusion (*P*=0.83). Day 60 RBAs (Figure 2) also indicated a difference between dietary treatments (*P*=0.01), with the 1.5% EXOP diet producing the greatest RBA (OD_{620}=0.73). The 1.5% inclusion diet was also greater than the BSBM control (*P*<0.01) and slightly greater than the FM reference (OD_{620}=0.67).

The analysis of phagocytic capacity showed variability over the course of the trial (Table 3). No differences in phagocytic rate was found on day 14 (P=0.31), day 28 (P=0.67), or day 42 (*P*=0.89). Day 60 rates were significant (*P*=0.01), with the 1% EXOP diet providing a greater phagocytic capacity than the FM (*P*=0.04), M\BSBM (*P*<0.01), and the 0.5% EXOP diet (*P*=0.01). Day 14 and day 42 results demonstrated lower phagocytic rates than day 28 and day 60. No significant difference in phagocytic rate was found from day 14 (P=0.31), day 28 (P=0.68, and day 42 (P=0.89). Day 60 rates were found to be significantly different (P=0.01), with the 0.5% inclusion having a greater phagocytic capacity than the FM reference (P=0.04), the BSBM control (P=0.01), and the 0% Diet (P=0.01).

No treatment differences were noted in lysozyme activity at day 14 (*P*=0.61; Figure 1), 28 (*P*=0.56; Figure 1), 42 (*P*=0.82; Figure 1) or day 60 (*P*=0.06; Figure 2). No differences in total Ig concentrations were found in day 14 (P=0.26), day 28 (P=0.69), day 42 (P=0.74) and day 60 (P=0.68) sera samples. (Figure 1; Figure 2). ACH50 values were not found to be different for day 0 (P=0.37), day 14 (P=0.81), day 28 (P=0.63), day 42 (P=0.74) and day 60 (P=0.23) sera samples (Figures 1 and 2). SSI results were significantly (P<0.001) increased in all diets containing EXOP (Figure 3). K-values were found to be reduced from BSBM levels in the 1%, 3%, and 6% EXOP treatments (P=0.01; Figure 4).

Discussion

Overall, results indicate that immunostimulation in yellow perch can be achieved using small inclusions levels of EXOP. The data also yielded information on innate immune parameters of head kidney macrophages, and their abilities to phagocytize and generate superoxide killing potential. The two-week sampling intervals for immune responses provided a snapshot of short-term and long-term immune potentiation. Phagocytic rate is often used as an investigatory immune parameter because it reflects the capacity for the head kidney macrophages to engulf pathogens which can be tied to immunostimulation, suppression, and disease resistance (Decostere et al. 2001, Harford et al. 2006). In this study, there was phagocytic variability throughout the study period, but significant differences were only found at Day 60, indicating there may be some long-term enhancements to phagocytosis at relatively low EXOP inclusions. EXOP addition did not appear to detract from the phagocytic competencies of the macrophages, in comparison to the FM and SPC control diets. There was also some evidence of immunopotentiation at day 60 sampling, with small EXOP inclusion levels enhancing superoxide production from head kidney macrophages. This trend is also confirmed with the increased phagocytosis rate at day 60 (1.5% inclusion). Interestingly, larger levels of EXOP did not appear to significantly reduce or increase immune parameters, indicating that smaller inclusion levels may allow for better phagocytosis rates. In future experiments, analysis of cytokine activity may also provide supporting evidence for increased head kidney macrophage function, as it has previously been observed that injected polysaccharides have the ability to upregulate IL-1β in the common carp (*Cyprinus carpio)* pronephros (Yuan et al. 2008).

The SSI is often implemented as a generalized measure of fish health, stress condition and immune status (Zapata et al. 1996). An increased SSI may be indicative of a stressed condition, overactive blood filtration, or inflammation (Hoole et al. 1996, Milla et al. 2010). However, in recent publications an elevated SSI may also be indicative of increased pathogen resistance (Hadidi et al., 2008). Thus, in future studies it would be beneficial to compare SSI levels within a pathogen challenge study to deduce any immunomodulation or disease tolerance. Furthermore, the differentiation in K values with increasing levels of EXOP warrants further investigation of growth metrics. Thus, there could be a balanced level of immune enhancement that may circumvent any potential complications from palatability, digestibility, or growth performance.

Immunological evaluation of yellow perch responses to treatment diets revealed changes in macrophage physiology at longer-term samplings. Inclusions levels of 1-1.5% elicited the highest RBA at day 42 through day 60, indicative of increased superoxide production by head kidney macrophages, and an enhanced potential to eliminate pathogens (Secombes, 1990; Sharp and Secombes, 1993). Furthermore, the 1% inclusion provided a more active phagocytic capacity, which is integral to enhanced pathogen clearance and activation of adaptive immunity. A previous soy-based feeding trial in rainbow trout *Oncorhynchus mykiss* found no significant difference in the long-term immunological status (phagocytosis rate and RBA for head kidney-derived macrophages) of trout fed various levels of commercial FSBM (Barnes et al. 2014), further validating the observed immunopotentiation from the addition of the EXOP. In further experiments, feeding duration and pulse-feeding should also be considered as factors when examine the fish response over longer intervals (Bricknell and Dalmo, 2005).

The serological evaluations indicated no modulation in lysozyme, alternative complement, or Ig concentrations. Recent studies in common carp have found that the oral administration of β-glucan increased complement and alternative complement levels following exposure to *Aeromonas salmoncida* (Pionnier et al. 2013; 2014). Thus, further challenge studies with bacterial pathogens may be useful in evaluating exopolysaccharide supplements.

In a commercial feed formulation, lowered inclusions may be beneficial to satisfy both growth and innate immunity enhancements in yellow perch. Storebakken (1985) found that some polysaccharides, included as aquafeed binders, reduced feed consumption and apparent digestibility coefficients in rainbow trout, indicating a need to further assess palatability and growth metrics before an optimal inclusion would balance both performance and immunological enhancements in a grower diet (Storebakken 1985). This graded-inclusion level trial demonstrates the need to develop higher quality BSBM products that would support immunological performance equivalent to or better than diets containing FM. Although there were some differences in condition factor at the higher EXOP inclusion levels, the BSBM did show The improvement of soy macronutrient composition, digestibility, palatability, and ultimate diet formulation through extrusion pre-treatments and bioprocessing optimization may better be elucidated through additional feeding trials and would complement these immunological findings when determining inclusion parameters.

There is potential for these novel soy aquafeed ingredients and bioprocessing streams to improve fish health for aquaculture producers. These feeding trial results have developed a baseline for designing further bioprocessed soy experimental diets to assess

immunostimulation and the incorporation of these exopolysaccharides may reduce the need to administer antibiotics for production stocks.

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Tables

Table 1. Diet formulations and predicted composition (g 100 g^{-1} dmb unless noted) of experimental diets, using fishmeal and bioprocessed soybean meal as main protein ingredients.

Designation	**P-54**	**P-55**	**P-56**	**P-57**	**P-58**	**P-59**	**P-60**
Constituent	**FM Ref**	**BSBM (No EXOP)**	**0.5% EXOP**	**1.0% EXOP**	**1.5% EXOP**	**3.0% EXOP**	**6.0% EXOP**
Fishmeal[a]	35.00	17.50	17.50	17.50	17.50	17.50	17.50
BSBM	0.00	19.84	19.84	19.84	19.84	19.84	19.84
EXOP	0.00	0.00	0.58	1.16	1.75	3.49	6.99
Dextrin	3.50	3.50	3.20	2.91	2.62	1.75	0.00
Empyreal 75	7.45	7.45	7.45	7.45	7.45	7.45	7.45
Wheat flour[b]	26.75	26.75	26.60	26.46	26.31	25.88	25.00
Wheat gluten[b]	9.05	9.05	9.08	9.11	9.14	9.23	9.41
CMC	1.16	1.16	1.16	1.16	1.16	1.16	1.16
Cellufil[c]	5.36	2.20	2.04	1.85	1.67	1.13	0.07
Vitamin premix[d]	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Mineral premix[e]	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Stay-C[f]	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Choline[g]	1.50	1.50	1.50	1.50	1.50	1.50	1.50
Arginine[g]	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Lysine[g]	0.80	0.80	0.80	0.80	0.80	0.80	0.80
Histidine[g]	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Sodium chloride[h]	1.20	1.20	1.20	1.20	1.20	1.20	1.20
Potassium chloride[h]	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Calcium phosphate[h]	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Calcium propionate[h]	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025
Fish Oil[i]	4.28	5.10	5.10	5.10	5.11	5.11	5.13
Soybean Oil[j]	2.05	2.05	2.05	2.05	2.05	2.05	2.05
Chromic Oxide[h]	0.50	0.50	0.50	0.50	0.50	0.50	0.50
totals	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Protein	42.0	42.0	42.0	42.0	42.0	42.0	42.0
Lipid	9.0	9.0	9.0	9.0	9.0	9.0	9.0
Fiber	7.3	4.7	4.6	4.4	4.2	3.6	2.6
Ash	13.5	10.4	10.4	10.4	10.4	10.4	10.4
NFE	25.0	30.5	30.7	30.9	31.1	31.7	32.9
Gross Energy (Kcal/kg)	4788	4825	4824	4824	4825	4824	4824
Protein:Energy Ratio (g protein/Kcal)	9.2	9.0	9.0	9.0	9.0	9.0	9.0
Energy:Protein Ratio (Kcal/g protein)	10.8	11.1	11.1	11.1	11.1	11.1	11.1

[a] Special Select, Omega Protein, Houston, TX; [b] Bob's Red Mill Natural Foods, Milwaukie, OR; [c] USB Corporation, Cleveland, OH; [d] ARS 702 premix, Nelson and Sons, Murray, UT; [e] SS #3 trace mix, Nelson and Sons, Murray, UT; [f] DSM Nutritional Products, Parsippany, NJ; [g] Pure Bulk, Roseburg, OR; [h] Fisher Scientific, Pittsburg, PA; [i] Virginia Prime Gold, Omega Protein, Houston, TX; [j] South Dakota Soybean Processors, Volga, SD. ***g(gram), Kcal (kilo calorie), kg (kilogram), EXOP (Exopolysaccharide)

Table 2. Ingredient composition and base mineral profile of dietary protein sources (g 100 g^{-1} dmb unless noted) incorporated in the yellow perch diets.

Proximates	Fishmeal	BSBM
Crude Protein	67.53	59.54
Crude Lipid	5.21	0.46
Crude fiber	0.18	2.96
Ash	25.33	7.04
Moisture (%)	6.48	3.91
Essential Amino Acids		
Arginine	3.69	3.90
Cysteine	0.50	1.00
Histidine	1.26	1.46
Isoleucine	2.73	2.90
Leucine	4.47	4.75
Lysine	4.58	3.60
Methionine	1.72	0.92
Taurine*	0.42	0.06
Threonine	2.32	2.35
Tryptophan	0.58	0.86
Valine	3.10	3.00

*Taurine analyzed as sulfonic acid

Table 3. Phagocytosis of head kidney-derived macrophages throughout trial sampling intervals.

Treatment	Day 14	Day 28	Day 42	Day 60
FM Reference*	0.139±0.009	0.499±0.024	0.142±0.017	0.326±0.045[b]
BSBM Control**	0.127±0.013	0.527±0.073	0.131±0.019	0.298±0.019[b]
0.5%	0.132±0.012	0.571±0.111	0.128±0.01	0.302±0.029[b]
1%	0.127±0.013	0.529±0.075	0.151±0.016	0.475±0.037[a]
1.5%	0.120±0.009	0.542±0.070	0.132±0.015	0.335±0.037[ab]
3%	0.173±0.021	0.557±0.052	0.133±0.008	0.360±0.033[ab]
6%	0.121±0.010	0.526±0.075	0.144±0.007	0.350±0.032[ab]

*FM (Fishmeal): **BSBM (Bioprocessed soybean meal)

Figures

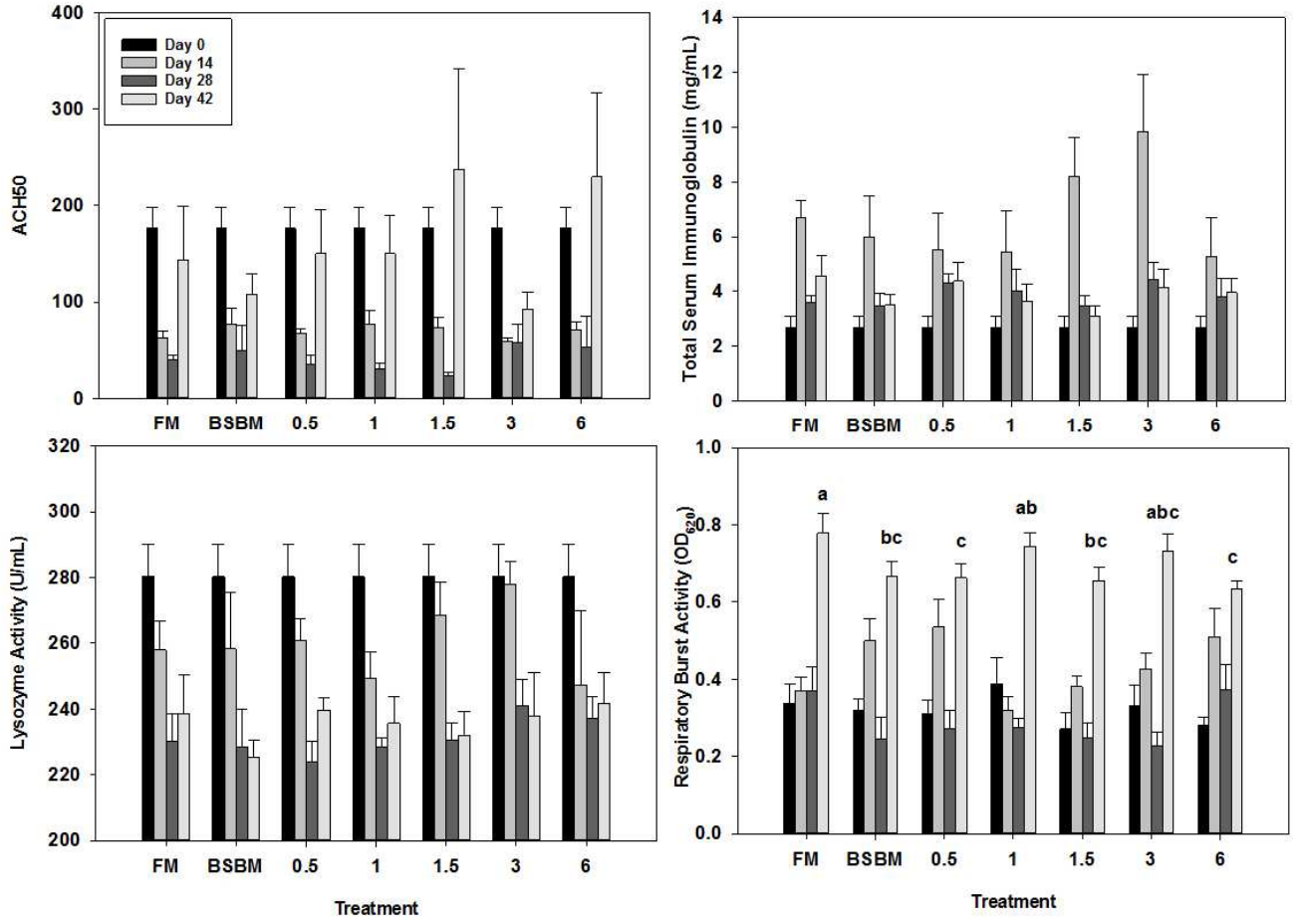

Figure 1. Respiratory burst activity (RBA), serum lysozyme, total immunoglobulin, and alternative complement activity (ACH50) of yellow perch sampled at days 0, 14, 28, and 42. Different letters denote significant differences among dietary treatments. FM=Fishmeal, BSBM =Bioprocessed soybean meal.

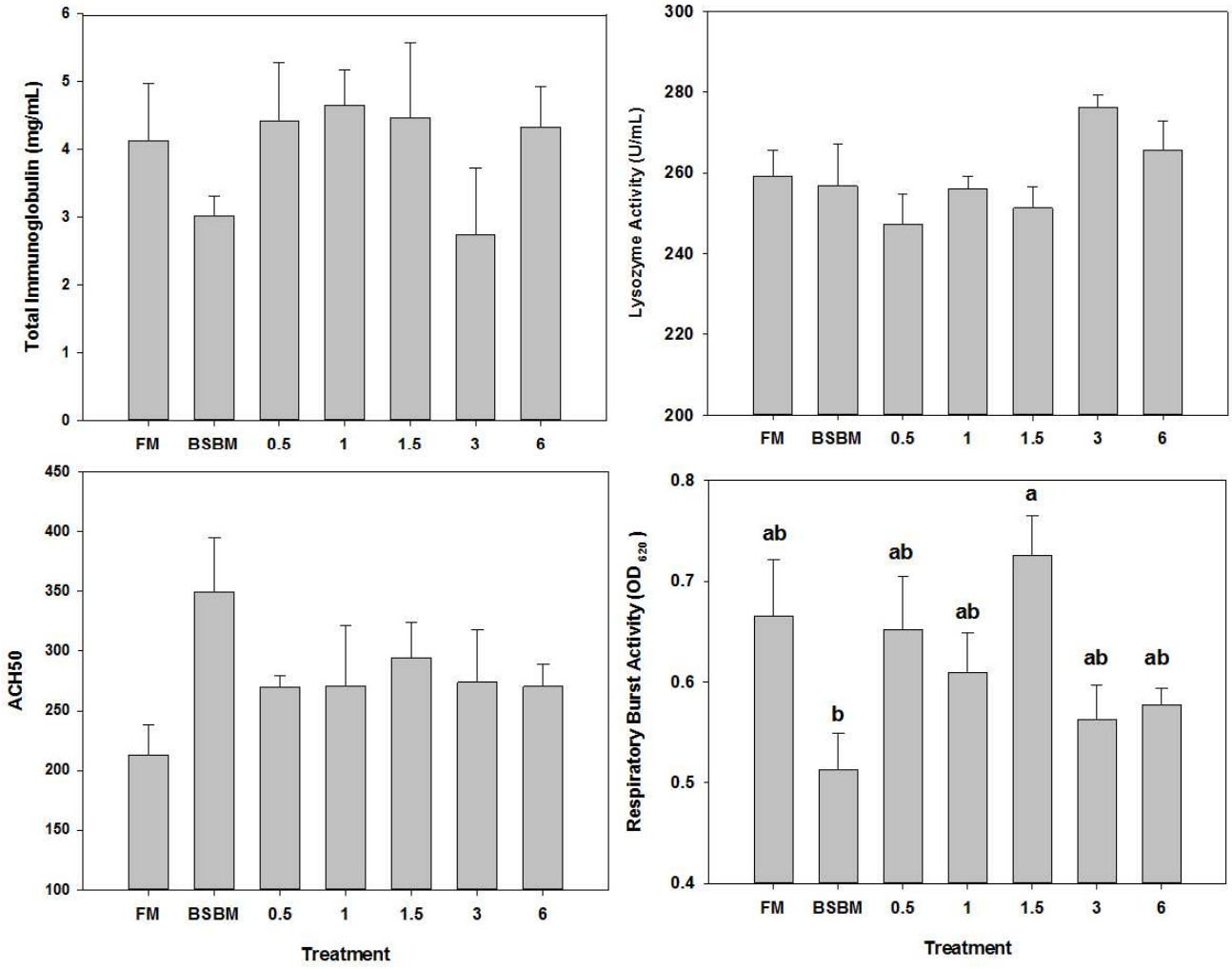

Figure 2. Respiratory burst activity (RBA), serum lysozyme, total immunoglobulin, and alternative complement activity (ACH50) of yellow perch sampled at day 60. Different letters denote significant differences between dietary treatments. FM=Fishmeal, BSBM =Bioprocessed soybean meal.

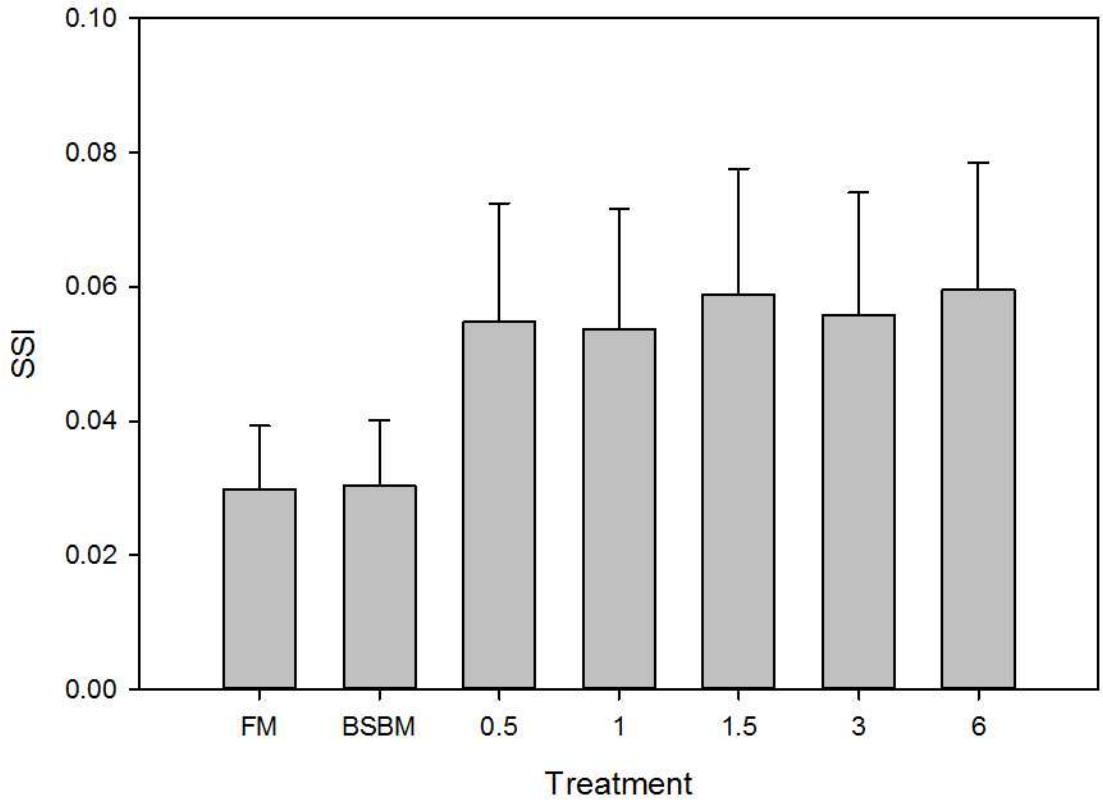

Figure 3. Spleen somatic index (SSI) of yellow perch sampled at day 60. FM =Fishmeal, BSBM=Bioprocessed soybean meal.

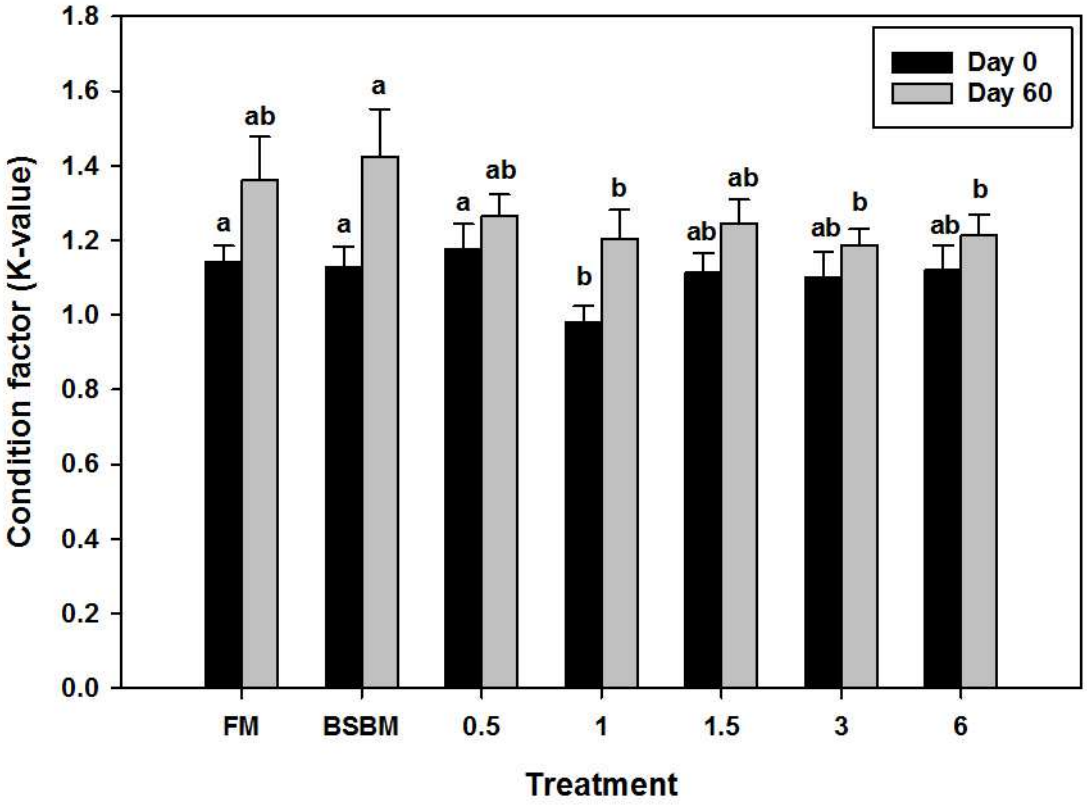

Figure 4. Fulton's condition factor (K-value) of yellow perch sampled at day 60. Different letters represent significant differences between treatments. FM=Fishmeal, BSBM =Bioprocessed soybean meal.

CHAPTER 3. CHARACTERIZATION OF INNATE IMMUNE PARAMETERS IN YELLOW PERCH (*Perca Flavescens*) FED BIOPROCESSED SOY-BASED DIETS SUPPLEMENTED WITH MINERALS AND POLYSACCHARIDES

Abstract

The availability of bioprocessed plant-based ingredients to replace marine-derived fish protein (MDFM) has gained momentum providing the potential for reducing feed cost and exploitation of wild fish stocks. Bioprocessed plant-based feedstuffs may also provide immunostimulatory enhancements, producing additional fish health benefits for aquaculture species. Minerals and polysaccharides have also been added to aquafeeds to augment fish immune responses such as macrophage activity, cytokine signaling, and antibody formation. A 30-day feeding trial with bioprocessed soybean diets supplemented with minerals (150 mg kg^{-1} and 300 mg kg^{-1} using zinc sulfate monohydrate; 2 mg kg^{-1} or 8 mg kg^{-1} using L-selenomethionine) or polysaccharides (β-glucans at 1.0% or 4.0%; commercial exo-polysaccharide at 3%) was performed with juvenile yellow perch (*Perca flavescens*). The study was conducted in a recirculating aquaculture system and fish were fed 1% of body weight daily. Yellow perch (54.03±1.76 g) were stocked in 30-gallon acrylic tanks at a density of 22 fish/tank. Fish were sampled on Day 7 and Day 25 to collect sera, head kidney (pronephros) macrophages, and spleens. On Day 25, dietary treatment fish were challenged with a lipopolysaccharide (LPS) intraperitoneal injection, or a phosphate-buffered saline (PBS) control, and sampled for immune metrics at 24h and 5d post-injection. Pronephros-derived macrophages were cultured and assessed for respiratory burst activity as

measured via superoxide production. Serum samples were assessed for alternative complement activity (ACH50), lysozyme content, and total immunoglobulin levels. Short-term respiratory burst activity (RBA) was significantly different at Day 7 (P=0.024), with the low inclusion of zinc producing greater superoxide levels (OD_{620}=0.548) than the commercial exo-polysaccharide (OD_{620}=0.326). At 5-days post challenge, there was no significant change in RBA between treatments in the LPS injected group (P=0.470), but the PBS control groups demonstrated some significant modulation (P<0.001) and the low-level β-glucan group demonstrated the greatest superoxide production (OD_{620}=0.567). Study results indicate a variation in short-term innate immune responses as a result of feeding mineral and polysaccharide supplemented bioprocessed soybean meals.

Introduction

The use of bioprocessed soybean meal in aquafeeds has increased as a fishmeal replacement in the aquaculture industry (Barnes et al., 2012). The processing mechanisms may be enzymatic, and / or microbial in nature, and some initial studies have sought to investigate the effect of these novel ingredients on teleost immune responses (Chen, 2013; Kokou, 2012). Similarly, mineral and vitamin supplements, as well as polysaccharide immunostimulants are also interest to fish health researchers, given that studies have demonstrated modulations to the fish immune system and pathogen resistance (Gannam & Schrock, 1999; Landolt, 1989). Elevated inclusion levels of mineral-containing compounds has been found to influence innate immunity, but tolerance limits and immunogenic effects vary among aquaculture species and their

nutritional requirements (Lim, 2001). Increased levels of selenium have been found to impact head-kidney macrophage function through the increased of intracellular superoxide production, while zinc has been found to have a mitogenic effect on carp lymphocytes *in vitro* (Ghanmi, 1993 ; Wise et al., 1993). Increased inclusion of immunostimulatory polysaccharides, such as β-glucans and mannan oligosaccharides (MOS), have also been found to increase innate immune parameters and cytokine production in fish species (Meena et al., 2013 ; Torrecillas, 2007 ; Chansue, 2000). β-glucan has also demonstrated an ability to reduce mortality against several fish pathogens including Aeromonas strains, and has also been noted to provide white spot syndrome virus (WSV) protection for cultured shrimp (Chang et al., 2013 ; Sajeevan et al., 2009). Combinations of bioprocessed soy diets with various levels of mineral or polysaccharides were applied to yellow perch, a species with commercial aquaculture potential, to evaluate inclusion rates and immune system responses (Shepherd et al., 2012 ; Rosauer et al., 2011 ; Brown, 2004).

Materials and Methods

Juvenile yellow perch (*Perca flavescens*) were obtained from the South Dakota Game, Fish and Park (Blue Dog State Hatchery, Waubay, SD) and stocked at rate of 22 fish per tank in a 32-tank (110L $tank^{-1}$) recirculating aquaculture system. Water quality parameters were monitored daily throughout the trial (21.9°C, pH 7.84, DO 7.57 mg L^{-1}) and the tanks were cleaned by siphoned daily. Prior to the start of the 30-day study, perch were acclimated to tanks and a reference diet for 7 days. Experimental diets were dispensed at Day 0 and fish were fed a ration equivalent to 1% body weight per day

(0.5% of body weight, twice daily). Three randomly selected fish per tank were collected at day 0 to provide baseline measurements (i.e., weight, length), and spleen, blood, and head kidney samples; all samples were pooled by tank to ensure adequate tissue collection. Yellow perch were sampled again on days 7 and 25 for immunological metrics. Immediately following the 25 day sampling, 8 fish per tank were challenged with an intraperitoneal (IP) injection of 5.0 mg kg^{-1} of purified lipopolysaccharide (*E. coli* 026:B6; Sigma Aldrich) in phosphate buffered saline (PBS; pH 7.2). An injection volume of 10 uL g^{-1} was adjusted for the average weight of each fish. A control group, 8 remaining fish within each tank, was also simultaneously injected with PBS at day 25, using the same IP injection volume. Challenged fish were then sampled at day 26 (Post-1) and day 30 (Post-5) to observe LPS-induced changes. The experiment was terminated following the day 30 sampling. Collected blood was allowed to clot at 4°C overnight in a 1.5mL centrifuge tube and then centrifuged at 1000 x g to isolate sera.

Diets were formulated on a dry matter, isonitrogenous and isolipidic basis (42% protein; 9% lipid; Table 1). The experimental bioprocessed soybean meal was produced by proprietary microbial or enzymatic bioprocesses (Prairie AquaTech, Brookings SD). Essential amino acids, vitamins, and minerals were supplemented equally in treatment diets to meet or exceed the known requirements for yellow perch (Hart et al., 2010; NRC 2011). Ingredients were analyzed using official methods for crude protein, crude fat, crude fiber, moisture, ash, and phytate (AOAC, 2006; Table 2). Furthermore, mineral concentrations of the soybean meal, fishmeal, and supplemental plant-based ingredients were verified by a commercial laboratory prior to formulation to assist with the accuracy of the inclusion levels. Zinc levels in treatments were adjusted to 150 mg kg^{-1} or 300 mg

kg^{-1} using zinc sulfate monohydrate (Acros Organics, Fair Lawn, NJ). Dietary selenium levels were adjusted to 2 mg kg^{-1} or 8 mg kg^{-1} using L-selenomethionine (Acros Organics, Fair Lawn, NJ). β-glucans was obtained from a commercial source and was incorporated at a 1% or 4% inclusion. Commercial exo-polysaccharide was obtained from a commercial source (Freda Biotechnology Corp, Shandong China) and was included at 3%.

Diet ingredients were prepared by grinding the large particles with a Fitzpatrick Comminutor (Elmhurst, IL) with 1.27 mm screen prior to dry blending. Dry ingredients were blended for 20 minutes using a V-10 mixer with an intensifier bar (Vanguard Pharmaceutical Machinery, Inc., Spring, TX). Dry blended feedstuffs were then transferred to a Hobart HL200 mixer (Troy, OH) where oils and water (30%, wb) were added and blended for ~5 minutes. Treatment diets were then screw-pressed under similar conditions using a Hobart 4146 grinder fitted with a variable speed feeder, variable speed knife drive, and 3.5mm die plate. Pellets were sieved and dried with a Despatch UDAF electric conveyor drier (Minneapolis, MN) with maximum temperature set at 120°C and retention time of ~15 minutes. Subsequently, the diets were cooled to room temperature and then stored at 4°C until used.

Spleen Somatic Index (SSI) and Fulton's condition factor (K-value) were calculated for fish sampled at day 0 and day 25 to compare any morphological changes over the duration of the feeding trial component. The following calculations were used:

$$\mathrm{K-value} = \frac{\text{final weight (g wet)}}{\text{total length (mm)}^{3}} \times 100{,}000$$

$$\mathrm{SSI} = \frac{\text{fresh spleen weight (g)}}{\text{whole fish weight (g wet)}} \times 100$$

Macrophages were isolated using previously established methods by Secombes et al. (1990), with modifications. Head kidney isolates were removed aseptically and stored on ice in 2mL of Leibovitz-15 (L-15) medium containing 2% fetal calf serum (FCS) (Sigma-Aldrich, St. Louis, MI), 100 i.u. penicillin-streptomycin mL^{-1} (Sigma-Aldrich), and 10 units of heparin mL^{-1} (Fisher Scientific, Waltham, MA). Head kidney samples were processed within 4h of extraction and the tissues were passed through 100μm cell screen mesh in 2mL of the ice cold, modified L-15 medium. The samples were then placed on a 34%/51% Percoll (Fisher Scientific, Waltham, MA) gradient and centrifuged at 500 x g for 30min. Cells were collected at the density interface and added to cold modified L-15 medium. The collected cells solutions were then centrifuged at 1000 x g for 10 min to wash residual Percoll and the pellet was re-suspended in L-15 supplied with 0.1% FCS. Samples were adjusted to $2x10^7$ cells mL^{-1} using a hemocytometer, verified for viability using trypan blue exclusion, and $2x10^6$ cells were seeded in each well of a 96 well tissue culture plate. Cells were allowed to adhere for 2h at 23°C before being washed with fresh L-15 (non-supplemented). Adherent cells were incubated overnight in L-15 supplemented with 5% FCS prior to being used in the assays. Blood was collected using a caudal vein sever, approximately equal parts were pooled for three fish per tank, and then deposited into sterile 2mL centrifuge tubes. The blood samples were allowed to clot at 4°C overnight and then centrifuged at 1000 x g for 10 minutes to isolate serum. Isolated sera were then frozen at -80°C in aliquots, pending assay.

Macrophage respiratory burst activity (RBA), derived from the pronephros was determined using spectrophotometric analysis of NBT reduction via superoxide

production. For the RBA reagent preparation, NBT was added at a concentration of 1mg mL^{-1} and 1μg mL^{-1} phorbyl 12-myristate 13-acetate was added to L-15 medium. 100μL of the RBA reagent was added to the wells and allowed to react for 30min. The wells were then emptied, the cells fixed with methanol, and the wells were washed twice with a 70% methanol rinse. 120μL of 2M potassium hydroxide (KOH) and 140μL of dimethyl sulfoxide (DMSO) were added to each well and the plates were agitated and read in a microplate reader (BioTek Corp., Winooski, VT) at 620nm, using KOH/DMSO wells as blanks. OD_{620} readings were adjusted for blanks and were compared with the respective RBA.

Serum lysozyme was detected using a turbidimetric assay with, lyophilized bacteria (*M. lysodeikticus*; Worthington Chemical, Lakewood, NJ) to determine inherent concentrations (Kim et al. 2006). Briefly, 100 ml of serially diluted serum in 0.05M sodium phosphate buffer (SPB; pH 6.2) in 96-well plates. 100 ml of 0.4 mg ml^{-1} suspension of the bacteria in SPB and the plates were agitated to mix samples. The optical density (O.D.) was measured at 570 nm at 0 and 15 min at 20°C. SPB was used alone as a microplate blank and replaced serum as a negative control. A unit of lysozyme activity was defined as the amount of serum causing a decrease in absorbance of $0.001min^{-1}$.

Total immunoglobulin (Ig) was determined using previously published methods (Siwicki et al 1993). Briefly, serum dilutions (1:32 and 1:64) were made in a 96-well plate with 0.85% saline solution, and bicinchoninic acid (BCA) protein assays (Pierce Biotechnology, Rockford, IL) were used to read total protein in the serum. 100μl of serum was added to 100μl of a 12% polyethylene glycol (PEG), incubated for 2h with

shaking, and centrifuged at 1000 x g to collect the supernatant. The supernatant was then processed using the BCA protein kit and absorbance from the PEG-treated sera was subtracted from the total protein calculation to provide the total immunoglobulin content.

Alternative complement activity (ACH50) was determined using the hemolysis of sheep red blood cells (RBCs) according to previously described and published methods (Welker et al. 2014). Sheep RBCs (10% packed volume in Alsever's solution; Lampire Biological, Pipersville, PA) were washed 4 times (300 x g at 4°C) with cold PBS with 1% gelatin (PBS+; pH 7.2) and suspended in PBS+. The cell solution was adjusted with a hemocytometer to a final concentration of $5x10^7$ RBC mL^{-1}. In a 96-well round-bottom plate, 50 µl of yellow perch sera were serially diluted in PBS+ (from 20% to 0.157% concentrations) and the volume diluted to 250µl. 50µl of the RBC solution was added to each well to bring the reaction volume to 300µl. Positive controls were used for each sample to represent 100% hemolysis (250µL dH_2O and 50µL RBCs). Negative controls were used for each sample to represent spontaneous lysis (250µL PBS and 50µL RBCs). Samples were incubated at 21°C for 1hr with agitation and the reaction was stopped by placing the plates on ice for 3 minutes. Samples were centrifuged (800 x g for 10min at 4°C) and 250µL of supernatant was transferred to a 96-well flat bottom plate. The absorbance (410nm) was recorded using a plate reader (EPOCH model; BioTek. Winooski, VT). Complement hemolytic activity is expressed as ACH50 units mL^{-1}, where a ACH50 unit represents the volume of serum necessary to produce the lysis of 50% of sheep erythrocytes. The degree of hemolysis was estimated via the lysis curve for $Y / (1 - Y)$ against the volume of serum added (µL). The Y value (percentage of

hemolytic activity at each dilution relative to the positive and negative controls) was determined by the equation:

$$Y = 100 \times [\text{Abs(A)} - \text{Abs(B)}] / [\text{Abs (C)} - \text{Abs (B)}]$$

Where, Abs (A) = absorbance of the yellow perch serum dilution; Abs (*B*)=absorbance of the negative control (spontaneous lysis); Abs (*C*) = absorbance of the positive control (100% RBC lysis).

Data analysis was completed using Program R (R Development Core Team, Vienna, Austria). Data were assessed normality using the Shapiro-Wilk test and homogeneous variances using Levene's test. Analysis of variance (ANOVA) was used to compare immune metrics, and Tukey's HSD was used for multiple comparisons. Non-normal data were subject to a square root transformation prior and analyzed using ANOVA, when applicable. Nonparametric data were analyzed with the Kruskal-Wallis one-way analysis by rank and multiple comparisons were computed using Mann-Whitney U tests with the Bonferroni correction to differentiate treatment differences. Results from statistical analyses were deemed significant at α=0.05.

Results

Day 7 RBA was found to be significant (P=0.024) between the low zinc and exo-polysaccharide treatments, while there was no difference among treatments with respect to the reference diet group (Figure 1). The high inclusion of β-glucans (OD_{620}=0.535) and zinc (OD_{620}=0.548) also produced greater superoxide levels than the reference diet (OD_{620}=0.522), although treatment differences were not significant. Day 25 macrophage

RBA was found to be significantly different across diets (P=0.001). The high selenium inclusion produced a significantly greater RBA than the high zinc inclusion (P=0.025), but was found to be less than the exo-polysaccharide diet (OD_{620}=0.393), which produced the greatest RBA. No differences in RBA were found within the LPS-challenged fish for the Post-1 (P=0.266) and Post-5 (P=0.470) samplings (Figure 2). The control fish had differences in RBA values at Post-1 (P=0.007) and Post-5 (P<0.001). At Post-1, the low selenium treatment within the control group showed the greatest RBA (OD_{620}=0.459) and was found to be greater than the control group's exo-polysaccharide treatment diet (P=0.020). At Post-5 within the control group, the low inclusion β-glucan treatment demonstrated the greatest RBA (OD_{620}=0.566) and was greater than the high level β-glucan diet (P<0.001).

The low inclusion β-glucan treatment resulted in the highest lysozyme content at day 7 (245.33 U mL^{-1}) and was different from the exo-polysaccharide treatment (196.67 U mL^{-1}; P=0.012). No differences were detected at day 25 (P=0.189), in the Post-1 LPS group (P=0.072), and in each of the LPS (P=0.143) and Control (P=0.055) groups at Post-5 (Figure 3). Treatment differences were detected in the Post-1 Control group, with the high-level inclusion β-glucan treatment producing a lysozyme content that was greater than the low zinc inclusion (P=0.022).

Differences in alternative complement activity were observed at Post-5 in the LPS group (P<0.001). The reference diet was found have a greater ACH50 than the high zinc (P=0.026) treatment, while the low zinc treatment did not display a difference in ACH50 (P=1.00; Figure 4).

No differences in antibody concentration were detected on day 7 (P=0.319), day 25 (P=0.180). Following the challenge, there was also no significance in levels for the Post-1 LPS (P=0.706) or Control (P=0.203), and Post-5 LPS (P=0.791) or Control (P=0.073) groups (Figure 5). There were no differences in SSI on day 0 (P=0.593) or day 25 (P=0.719). K-value also did not differ on day 0 (P=0.612) or day 25 (P=0.100).

Discussion

The ability of cultured fishes to properly eliminate bacterial pathogens is of major importance to fish producers as it may reduce the need to treat large populations with antibiotics and therapeutants (Blanco, 2000). A firm understanding of the effects of dietary ingredient enhancements on the fish immune system will better characterize immune interactions and allow inclusion rates of these products to benefit commercial applications. Furthermore, immunostimulants may have various periods of functional protection, and this may be dependent on species and the ontogeny of the immune system (Bricknell & Dalmo, 2005; Sakai, 1999). Study results indicate that mineral and polysaccharide immunostimulants have the ability to modulate the fish immune system, and furthermore, diet-induced differences were observed between LPS-challenged and unchallenged yellow perch, although dietary treatments differences were not always found to be immunopotentiating.

For example, macrophage RBA activity was reduced in the LPS-injected fish at both Post-1 and Post-5 samplings, while the control groups demonstrated a greater RBA among treatment diets. Although this LPS induced reduction in RBA appears to contradict recent findings where increased phagocytosis and superoxide production

followed LPS administration, a RBA reduction in European sea bass (*Dicentrarchus labrax*) was noted in a previous study by Sarmento et al. (2004). Similarly, Nayak et al. (2008) found that small doses (1-2 EU) of *E. coli* LPS increased RBA in macrophages in an immunostimulatory manner, while larger doses (10-20 EU) appeared to be immunosuppressive. Thus, further studies with graded inclusion levels of β-glucans that are lower than the range utilized in this experiment may better determine the optimal dosing levels, specifically for yellow perch.

Both zinc supplemented diets demonstrated a similar RBA when compared to the reference diet, results that are corroborated in European sea bass fed 148 mg kg^{-1} zinc oxide (Fountoulaki et al., 2010). Although zinc may not greatly influence RBA at short-term intervals (150 mg kg^{-1} and 300 mg kg^{-1} dosing), these zinc sulfate inclusions may cause a reduction in RBA following 25 days of feeding in yellow perch. Comparatively, the selenomethionine supplemented diets did not differ from the reference diet for RBA levels. From previous studies, selenium deficiencies appear to reduce the integrity of innate immunity and these findings may explain the lack of significance in RBA activity in fish fed both high and low-level selenium treatment diets (Webster, 2015). Thus, further experimentation using selenium deficient diets may be useful for better discerning treatment effects with selenium inclusion rates.

It has been found that lysozyme production increases in response to LPS and results from this study corroborate the increase in lysozyme production across all LPS-challenged perch, except for the high inclusion of β-glucans, at Post-1 sampling (Paulsen et al., 2003). Paulsen et al. (2001) also reported that increasing concentrations (0.1-250 μg mL^{-1}) of particulate β-glucans in the isolated macrophage (rainbow trout) supernatant

was correlated with increased lysozyme production. The oral administration of the β-glucan in the present study versus the *in vitro* particulate administration to cultured macrophages may account for the slight decrease of lysozyme activity. The LPS (4% β-glucan) group was demonstrated decreased serum lysozyme compared to the PBS-injected (4% β-glucan) control group. Furthermore, it is known that LPS exerts its immunomodulatory effect through the activation of Toll-like receptor 4b (TLR4b), while β-glucans interacts with TLR2 to stimulate the innate immune system (Palti, 2011; Tanekhy, 2016; Bricknell & Dalmo, 2005). Thus, co-stimulation or interactions between these TLRs or downstream pathways may also have exerted an effect on the head-kidney macrophage populations.

The total immunoglobulin was found to be elevated in both Post-1 and Post-5 LPS-challenged perch, but there were no significant treatment effects at these sampling timepoints. This response aligns with previous findings from LPS challenge studies in both carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) (Nakhla et al., 1997 ; Kozinska, 2004). ACH50 values were depressed across the control group treatment diets, with the exception of the BSBM reference diet and high zinc inclusion. This contradicts recent findings by Pionnier et al. (2013) where carp fed β-glucans demonstrated increased alternative complement activity in the sera and a two-fold increase in complement reactive protein levels. Furthermore, in a later study, results indicated no increase in complement activity in the β-glucans group, while the control diet appeared to have elevated CRP levels and complement activity (Pionnier et al., 2014). That study also highlighted some changes in *crp2* gene expression within treatment groups. As alternative complement activity is independent of antibody response

and directly activated by LPS, further investigation of species-specific gene expression in yellow perch may better elucidate the molecular mechanisms for alternative complement dynamics with respect to added immunostimulants (Boshra et al., 2006).

This challenge study incorporated multiple dietary immunostimulants and dosing levels along with bioprocessed soybean meal. The results from this research effort are applicable to yellow perch producers and researchers evaluating diet formulations and attempting to maintain healthy stocks. Immunostimulants may provide value-added therapeutants to aquaculture production and bolster vaccine efficacy through adjuvant functions and allow for potential diet-related enhancements to innate immunity (Tafalla et al., 2013; Siwicki et al., 1994).

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Tables

Table 1. Diet formulations and predicted composition (g 100 g^{-1} dmb unless noted).

Constituent	Reference	Se-H	Se-L	Zn-H	Zn-L	EXOP	βG-H	βG-L
Fishmeal[a]	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
BSBM	33.00	33.00	33.00	33.00	33.00	33.00	33.00	33.00
Dextrin	5.70	5.70	5.70	5.70	5.70	2.70	1.70	4.70
Empyreal 75	8.69	8.69	8.69	8.69	8.69	8.69	8.69	8.69
Wheat flour[b]	27.00	27.00	27.00	27.00	27.00	27.00	27.00	27.00
Wheat gluten[b]	3.55	3.55	3.55	3.55	3.55	3.55	3.55	3.55
CMC	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Cellufil[c]	0.06	0.06	0.06	0.00	0.04	0.06	0.06	0.06
Vitamin premix[d]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Mineral premix[e]	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Micro-ingredients	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
β-glucans	-	-	-	-	-	-	4.00	1.00
Exo-polysaccharide	-	-	-	-	-	3.00	-	-
Selenomethionine	-	0.0018	0.0005	-	-	-	-	-
Zinc Sulfate	-	-	-	0.062	0.0188	-	-	-
Fish oil[i]	5.85	5.85	5.85	5.85	5.85	5.85	5.85	5.85
Soybean oil[j]	1.69	1.69	1.69	1.69	1.69	1.69	1.69	1.69
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Protein	42.0	42.0	42.0	42.0	42.0	42.0	42.0	42.0
Lipid	9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0
Fiber	5.21	5.21	5.21	5.15	5.19	5.21	5.21	5.21
Ash	7.58	7.08	7.08	7.11	7.09	7.08	7.08	7.08
NFE	32.42	32.42	32.42	32.42	32.42	32.12	28.42	31.42
Gross Energy (Kcal/kg)	4765	4765	4765	4762	4764	4752	4600	4724

[a] Special Select, Omega Protein, Houston, TX; [b] Bob's Red Mill Natural Foods, Milwaukie, OR; [c] USB Corporation, Cleveland, OH; [d] ARS 702 premix, Nelson and Sons, Murray, UT; [e] SS #3 trace mix, Nelson and Sons, Murray, UT; [f] DSM Nutritional Products, Parsippany, NJ; [g] Pure Bulk, Roseburg, OR; [h] Fisher Scientific, Pittsburg, PA; [I] Virginia Prime Gold, Omega Protein, Houston, TX; [j] South Dakota Soybean Processors, Volga, SD.

Table 2. Ingredient composition and base mineral profile of dietary protein sources (g $100g^{-1}$ dmb unless noted) incorporated in the yellow perch diets.

Proximates	Fishmeal	BSBM
Crude Protein	67.53	69.68
Crude Lipid	5.21	0.86
Crude fiber	0.18	5.11
Ash	25.33	4.70
Moisture (%)	6.48	8.71
Essential Amino Acids		
Arginine	3.69	4.00
Cysteine	0.50	1.00
Histidine	1.26	1.50
Isoleucine	2.73	3.00
Leucine	4.47	4.88
Lysine	4.58	3.70
Methionine	1.72	0.95
Taurine*	0.42	0.06
Threonine	2.32	2.42
Tryptophan	0.58	0.88
Valine	3.10	3.10
Minerals		
Calcium	4.76	0.31
Phosphorous	3.01	0.64
Potassium	1.06	1.64
Magnesium	0.21	0.25
Sodium	0.74	0.01
Copper (mg kg^{-1})	4.30	24.08
Iron (mg kg^{-1})	801	139.22
Manganese (mg kg^{-1})	44.1	35.59
Selenium (mg kg^{-1})	2.2	1.23
Zinc (mg kg^{-1})	97.8	41.53

Figures

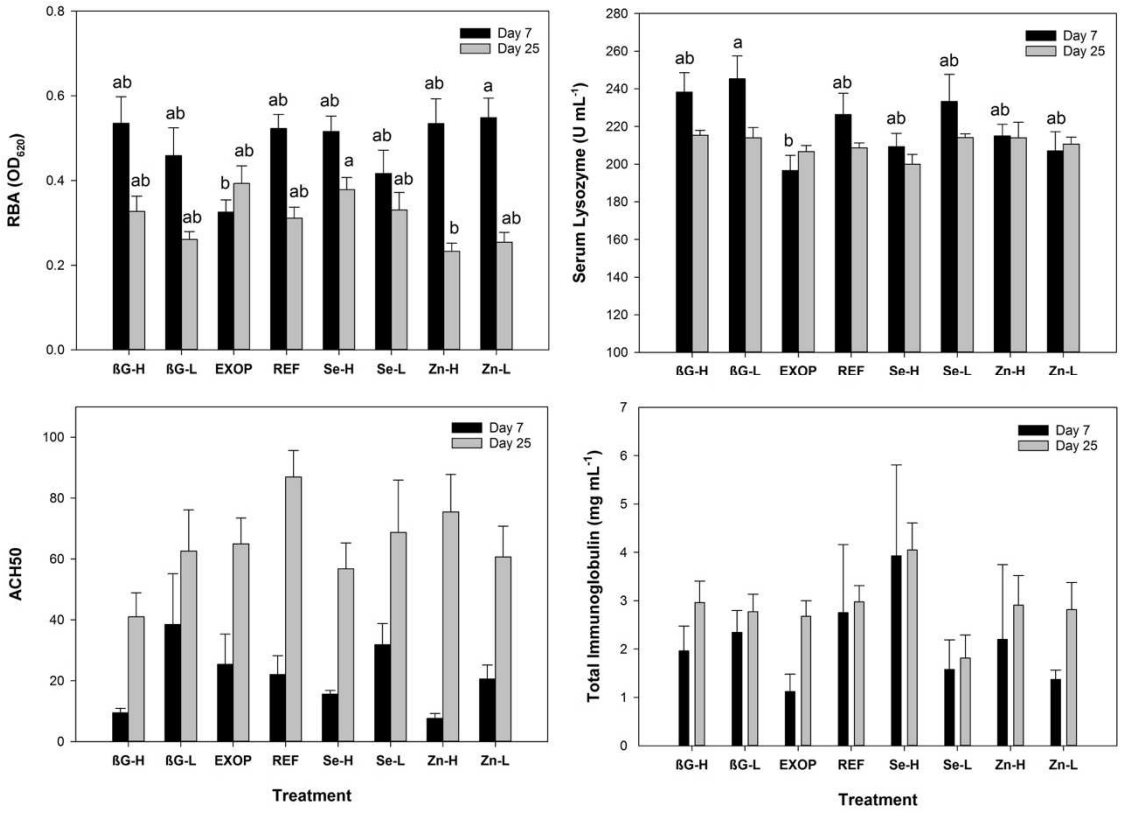

Figure 1. Respiratory burst activity (RBA), serum lysozyme, total immunoglobulin, and alternative complement activity (ACH50) of yellow perch sampled at day 7 and 25. Different letters denote significant differences between dietary treatments. βG=Beta glucans, Zn=Zinc, Se= Selenium, EXOP= Exo-polysaccharide, REF=Reference diet, H=High inclusion, L=Low inclusion.

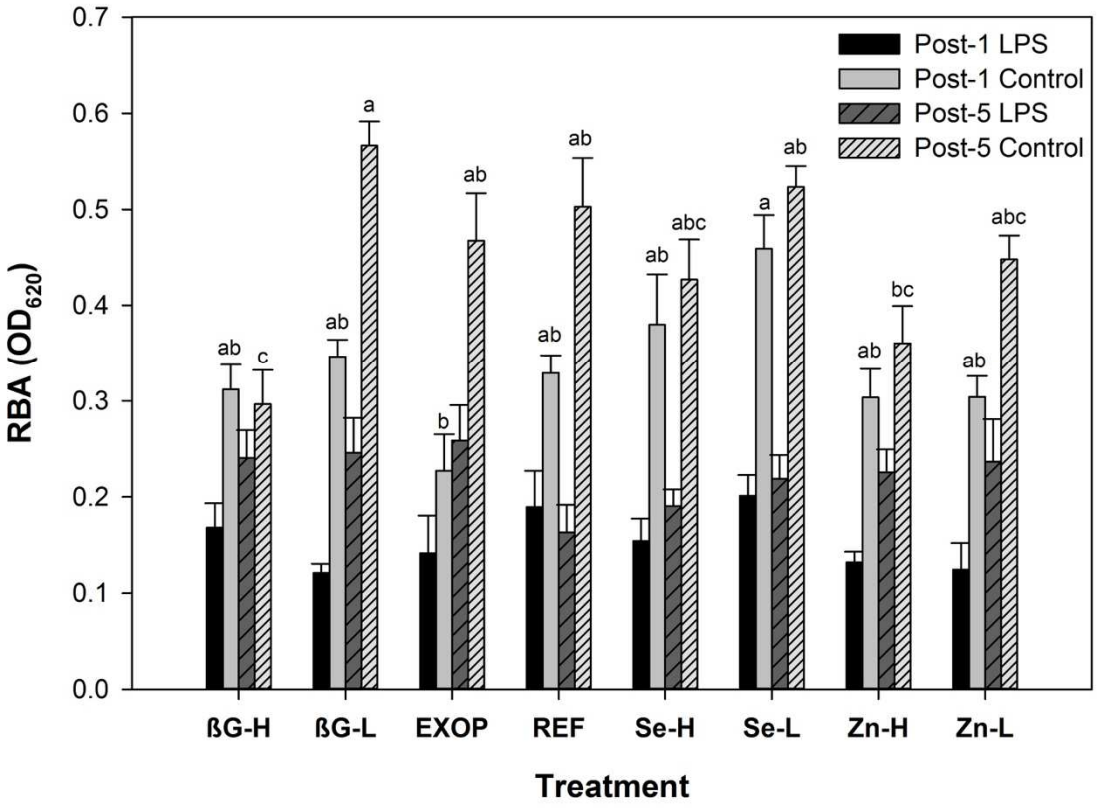

Figure 2. Respiratory burst activity (RBA) of yellow perch at post-challenge day Post-1 and day Post-5. Different letters denote significant differences between dietary treatments. βG=Beta glucans, Zn=Zinc, Se= Selenium, EXOP= Exo-polysaccharide, REF=Reference diet, H=High inclusion, L=Low inclusion.

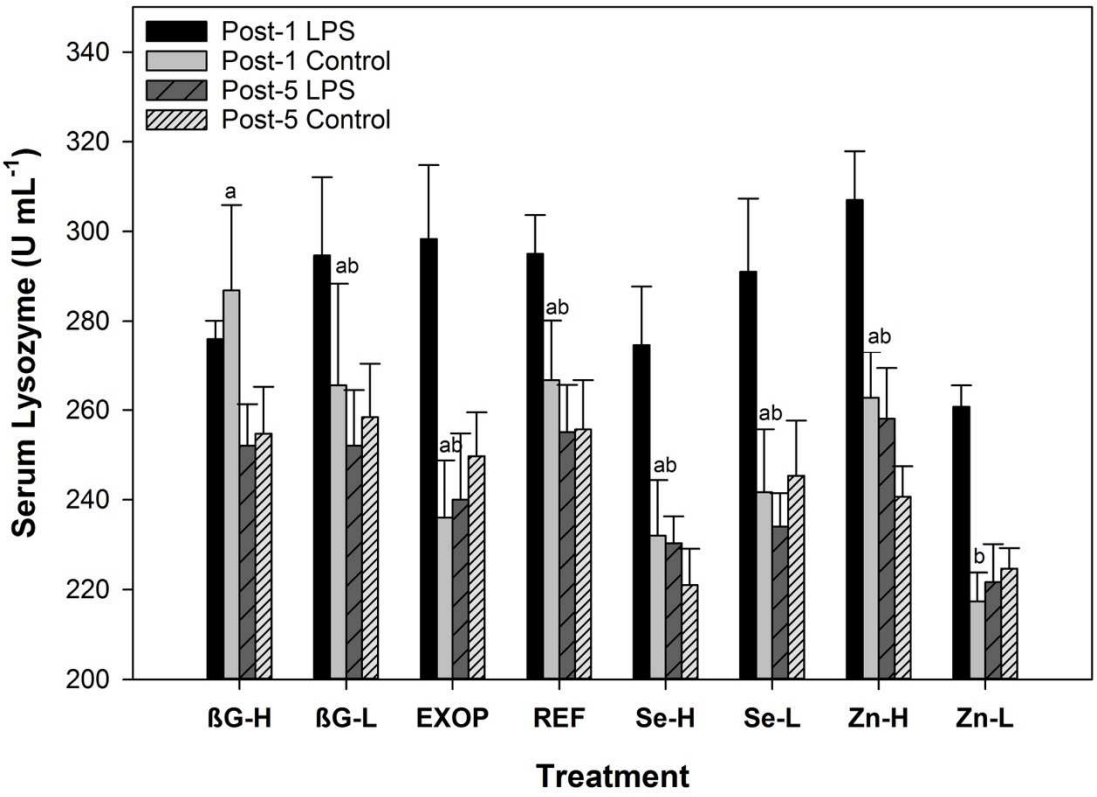

Figure 3. Serum lysozyme content of yellow perch at post-challenge day Post-1 and day Post-5. Different letters denote significant differences between dietary treatments. βG=Beta glucans, Zn=Zinc, Se= Selenium, EXOP= Exo-polysaccharide, REF=Reference diet, H=High inclusion, L=Low inclusion.

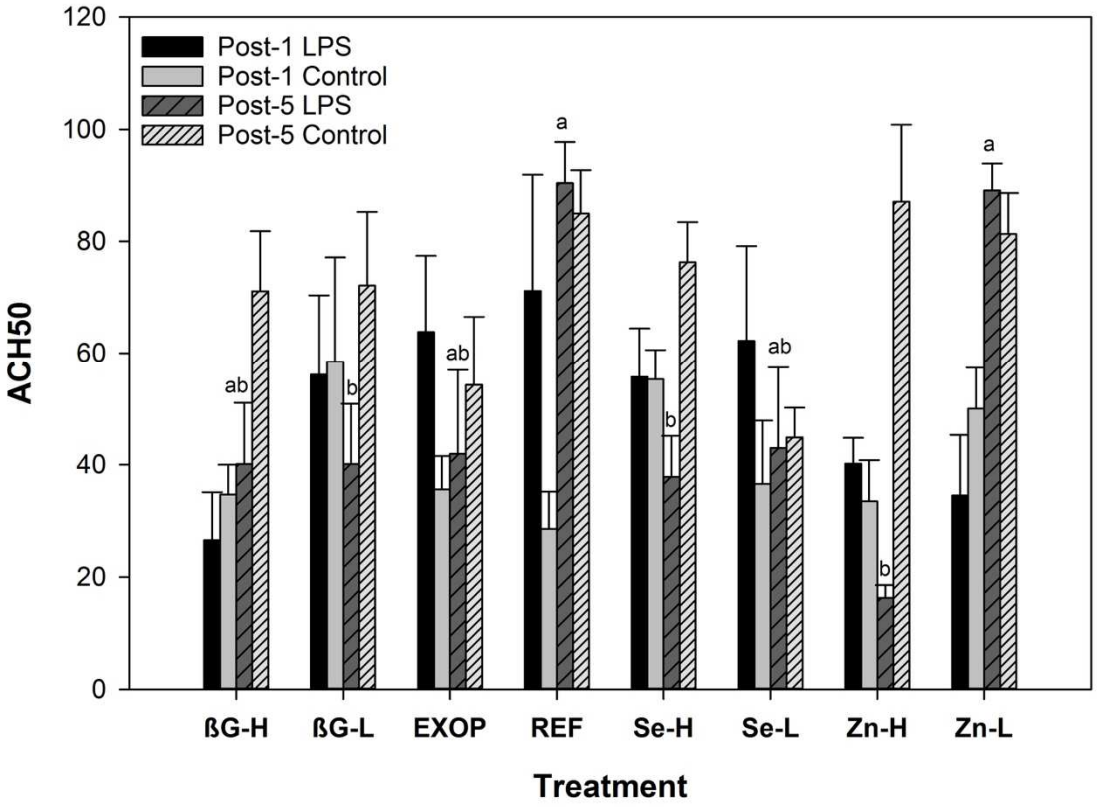

Figure 4. Alternative complement activity (ACH50) of yellow perch at post-challenge day Post-1 and day Post-5. Different letters denote significant differences between dietary treatments. βG=Beta glucans, Zn=Zinc, Se= Selenium, EXOP= Exo-polysaccharide, REF=Reference diet, H=High inclusion, L=Low inclusion.

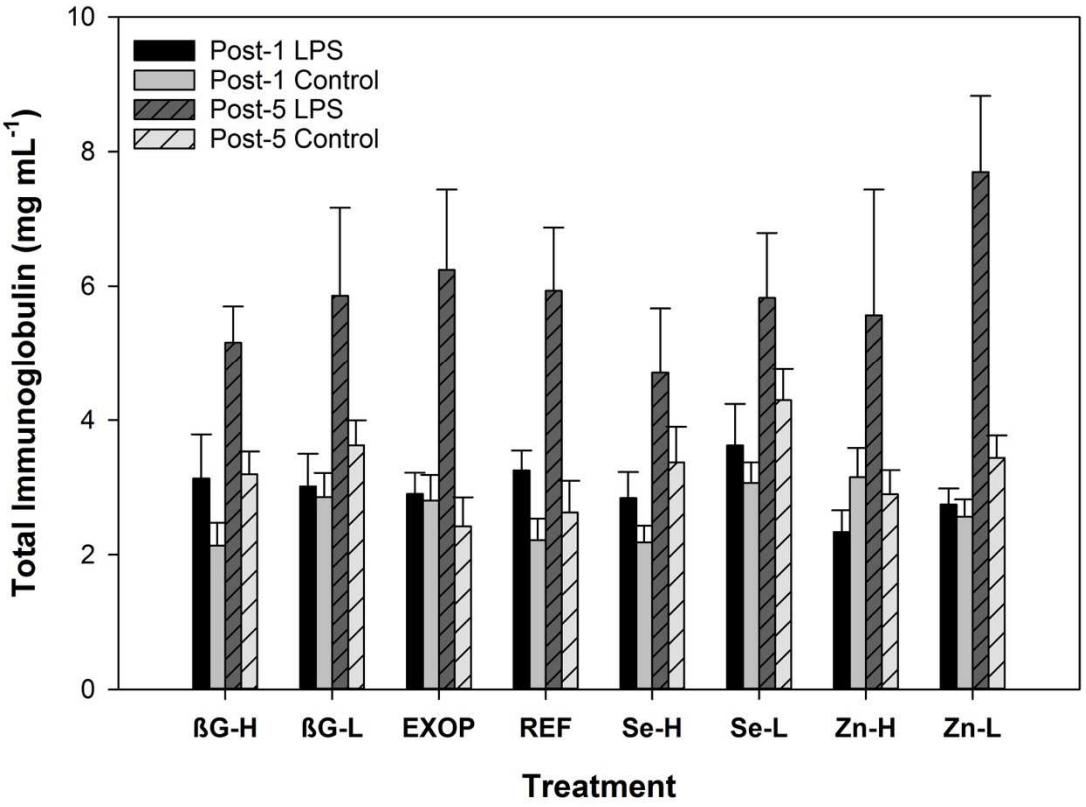

Figure 5. Total immunoglobulin content in sera of yellow perch at post-challenge day Post-1 and day Post-5. Different letters denote significant differences between dietary treatments. βG=Beta glucans, Zn=Zinc, Se= Selenium, EXOP= Exo-polysaccharide, REF=Reference diet, H=High inclusion, L=Low inclusion.

CHAPTER 4. IMMUNOMODULATION IN RAINBOW TROUT *Oncorhynchus mykiss* FED BIOPROCESSED SOY DIETS AND CHALLENGED WITH *Flavobacterium psychrophilum*

Abstract

Plant-based feedstuffs used as fishmeal (FM) protein replacements have the potential for reducing feed costs and providing comparable performance in aquaculture stocks. Antigenic components may be present in the bioprocessed ingredients following these microbial modifications. Thus, there may be a potential for immunostimulatory enhancements and the ability to enhance the immune response following exposure to aquaculture pathogens. A 79-day feeding trial incorporating bioprocessed soybean meal (BSBM) diets supplemented with a microbial was performed with juvenile rainbow trout (9.6±0.6g). The study was conducted in a biosecure recirculating aquaculture system and trout were fed two BSBM diets supplemented with exo-polysaccharide (EXOP) or β-glucans (βG). On day 69, trout (39.8±0.8g) were challenged with 100µl of inoculum (72h in TYES; OD_{525}=0.500) via intraperitoneal injection with *F. psychrophilum* isolate. Treatment fish were subsampled on day 0, 46, 60, and 79 to collect sera, head kidney macrophages, and organs. Pronephros-derived macrophages were cultured and assessed for respiratory burst activity (RBA), and organs were harvested to assess cytokine levels organ histology, respectively. Serum samples were assessed for alternative complement activity and lysozyme content. Bacterial plate cultures were re-isolated from challenged fish or mortalities to confirm *F. psychrophilum* infection using MALDI-TOF. TNF-α and IL1β gene expression were examined in head kidney and spleen tissues and

histological examination was performed during the post-challenge sampling to examine potentially malignant organs. At day 46, the RBA of the supplemented bioprocessed soybean meal variant 1 (BSBM1) treatments performed statistically better than the FM control (P=0.009 and P=0.002). The BSBM-1 base diet (OD_{620}=0.407) was also found to elicit a greater RBA (OD_{620}=0.373) than the control diet, although not significantly different. The supplemented BSBM-1 diets also generated a greater RBA than the supplemented bioprocessed soybean meal variant 2 (BSBM-2) diets (P=0.004 for exo-polysaccharide and P=0.020 for the β-glucans). Survival was highest in the β-glucans treatment (59.53%) while the fish fed BSBM-1 (41.07%) and fishmeal control diet demonstrated the lowest survival (40.55%). Findings indicate immunomodulatory responses as a result of feeding supplemented bioprocessed soybean meals.

Introduction

The incorporation of plant-based feedstuffs as a fishmeal replacement in aquaculture has gained momentum as fishmeal prices fluctuate and bioprocessing advancements create plant-based protein products with enhanced nutritional profiles (Chen, 2013; Olsen, 2012; Hardy, 2010). New research efforts have aimed to investigate the influence of these bioprocessed plant-based proteins on fish health, in addition to nutritional enhancements for rearing performance (Yang et al., 2015; Kokou, 2012; Kim, 2009). In addition to the immunological evaluation of these enhanced plant-based proteins, the incorporation of dietary immunostimulants, such as β-glucans, mannan oligosaccharides (MOS), and algal extracts have also become of interest to aquaculture health researchers (Guzman-Villanueva et al., 2014; Song et al., 2014; Gannam &

Schrock, 1999). These immunostimulants have the ability to augment the humoral and cellular defense mechanisms in cultured fishes, thus providing immunomodulation within the innate immune system (Bricknell & Dalmo, 2005). These immunostimulants have also been used to evaluate immune parameters in challenge trials with bacterial fish pathogens (Refstie et al., 2010 ; Selvaraj et al., 2005).

Flavobacterium psychrophilum, the causative agent of bacterial cold water disease (BCWD) is a gram-negative bacterium that can cause economic losses to salmonid aquaculture production (Barnes, 2011). The bacteria gains entry though the epithelium and is commonly characterized by a blackened skin coloration in the caudal region, skin ulcerations from the bacterially-associated metalloproteases, and erratic swimming behavior (Barnes, 2011; Wiklund & Dalsgaard, 2003; Secades 2003). Salmonid production losses of up to 25% have been reported in some U.S. states and vaccines have not been commercialized for widespread use in aquaculture (Van Vliet et al., 2015; Gomez et al., 2014). Salmonid culturists have incorporated management strategies, breeding programs, and manipulations to rearing conditions in an effort to curb losses resulting from *F. psychrophilum* hatchery outbreaks (Weber et al., 2013; Sugahara & Eguchi, 2012).

A long-term feeding and challenge trial was conducted to examine the effects of bioprocessed soybean meals and added immunostimulants on immune mechanics in rainbow trout infected with a *F. psychrophilum*. The study aimed to characterize changes to innate immune attributes, cytokine gene expression, and overall survival following exposure to an uncharacterized pathogenic *F. psychrophilum* strain isolated from a state

hatchery. Feeding trial results may elucidate and bolster nutritional-related management strategies against BCWD.

Materials and Methods

Feeding Trial Design

The 79-day experiment took place in biosecure laboratory (BSL). Five hundred and sixty juvenile rainbow trout (9.56±0.56 g) were randomly stocked into 40, 38L tanks at a density of thirteen fish per tank. Five diets were formulated to include fishmeal and a bioprocessed soybean meal (BSBM) as main protein sources (Table 1). Each dietary treatment was allocated to 8 replicate tanks for the study duration. The study tanks received replacement well water and the recirculating aquaculture system (RAS) was maintained at optimal conditions for rainbow trout culture (15°C, 7 mg L^{-1} dissolved oxygen, pH 7.65). Fish were fed by hand twice daily to satiation (approximately 1-2% of body weight). Tanks were siphoned daily and mechanical filtration units were also cleaned daily to remove fecal material. No palatability or feeding issues were detected across any of the dietary treatments during the trial duration and fish were found to be healthy during the entire pre-trial.

Five isonitrogenous and isolipidic diets were formulated to contain 42% protein and 18% lipid (Table 2; Table 3) and select diets were supplemented with polysaccharides to evaluate potential immunostimulatory effects. A fishmeal control diet (FMC) contained 20% menhaden fishmeal (Special Select, Omega Prime, Houston, TX) as the primary protein source, while the other two diets contained only 5% menhaden fishmeal, providing approximately 75% menhaden fishmeal replacement. A proprietary

microbial bioprocess (Prairie AquaTech, Brookings,SD) was used to manufacture the BSBM protein. β-glucans was obtained from a commercial supplier and was incorporated at a 0.1% inclusion. Commercial exo-polysaccharide was obtained from a commercial source (Freda Biotechnology Corp, Shandong, China) and was included at 1%. Essential amino acids, vitamins, and minerals were supplemented equally in treatment diets to meet or exceed the known requirements for rainbow trout (National Research Council, 2011). Ingredients were analyzed using official methods for crude protein, crude fat, crude fiber, moisture, ash, and phytate (AOAC, 2006; Table 2). Diet ingredients were prepared by grinding the large particles with a Fitzpatrick Comminutor (Elmhurst, IL) with 1.27 mm screen prior to dry blending. Dry ingredients were blended for 20 minutes using a V-10 mixer with an intensifier bar (Vanguard Pharmaceutical Machinery, Inc., Spring, TX). Dry blended feedstuffs were then transferred to a Hobart HL200 mixer (Troy, OH) where oils and water were added and blended for approximately 5 min. Treatment diets were then screw-pressed under similar conditions using a Hobart 4146 grinder fitted with a variable speed feeder, variable speed knife drive, and 3.5mm die plate. Pellets were sieved and dried with a Despatch UDAF electric conveyor drier (Minneapolis, MN) with maximum temperature set at 120°C and retention time of approximately 15 minutes. Subsequently, the diets were cooled to room temperature and then stored at 4°C until used.

Following an acclimation period of 7 days, 24 fish that were additionally stocked into excess tanks within the RAS were removed and assessed for baseline metrics, including sera collection and macrophage activity. 3 fish per tank were again sampled on days 46 and 60 to assess innate immune parameters. During the pre-challenge sampling

periods (days 46 and day 60), sera, head kidneys, spleens, and gross body measurements were collected for analysis. The fish remained on the same treatment diets and feeding schedule until the *F. psychrophilum* challenge at day 69.

F. psychrophilum inoculum preparation and challenge

Rainbow trout were challenged at day 69 with a reconstituted strain of *F. psychrophilum* (17830) isolated from a South Dakota trout hatchery. A challenge protocol was compiled using previously published methodologies, as listed below. First, an isolated *F. psychrophilum* colony from the original stock culture was removed and added to 10ml of tryptone yeast extract broth with salts (TYES; 0.4% tryptone, 0.04% yeast extract, 0.05% calcium chloride dihydrate, 0.05% magnesium sulphate heptahydrate, pH 7.2 in dH2O) and inoculated under sterile conditions in biosafety cabinet (BSC). Following 24h growth at 15°C, 1mL of inoculum was added to 200mL of fresh TYES broth in a 500mL flask. A sample of the 24h growth was back-plated on TYES for purity, and then confirmed by matrix-assisted laser desorption/ionization time-of-flight analysis (MALDI-TOF). Following a 72h incubation period the bacteria was pelleted by centrifuging the broth at 1200 x g at 15°C for 10 min (Madsen, 1999). Bacterial cells were resuspended in 0.85% saline to an OD (525nm) of 0.500 (estimated equivalent of $7x10^7$ CFU mL^{-1} and verified by a Biotek EPOCH microplate reader (LaFrentz et al., 2002 ; Holt, 1987). 100µl of challenge inoculum was plated on TYES agar plates and the growth observed for 7 days to observe culture purity (Cain & Lafrentz, 2007).

Juvenile rainbow trout were injected with the freshly prepared inoculum via intraperitoneal (IP) injection with a 1cc, 25ga insulin syringe (Becton Dickinson Co, Franklin Lakes, NJ). Rainbow trout weight was estimated from the day 60 sampling (average weight of approximately 35g) in an effort to estimate inoculum and dosage needs, and tank weights were collected prior to injection to determine actual tank biomass at the time of challenge (actual size of 39.8±0.8g). An injection volume of 100µl, based on the Madsen et al. (1999) studies, where 28g trout were injected with 50µl inoculum volumes. Fish were anesthetized using a 40mg L^{-1} tricaine methanesulfonate (MS-222) and placed in recovery bath with salt following injection. Following the IP injection, fish were fed treatment diets *ad libitum* and tanks were checked twice daily for mortalities.

At post-challenge day 10, the challenge trial was terminated. 3 fish per tank were sampled for spleens, blood, and head kidneys were extracted for macrophage analysis. Swabs were taken from the gills, external epithelium and abdominal cavity and plated on TYES agar plates to confirm infectivity with *F. psychrophilum*. The re-isolated plates were incubated for 7 days and then *F. psychrophilum* was confirmed via MALDI-TOF. Further, spleens, kidneys and gill samples from 20 infected fish (randomly sampled across all dietary treatments) were placed in phosphate-buffered formalin for qualitative histological examination. Slides were then prepared using H&E staining and observed under a 100x objective lens on a Nikon E-200 microscope (Nikon Corporation, Melville, NY).

Macrophage Respiratory Burst Activity

Macrophages were isolated using established methods by Secombes et al (1990), with modifications (Secombes, 1990). Head kidney isolates were removed aseptically and stored on ice in 2mL of Leibovitz-15 (L-15) medium containing 2% fetal calf serum (FCS) (Sigma-Aldrich, St. Louis, MI), 100 i.u. penicillin-streptomycin mL^{-1} (Sigma-Aldrich), and 10 units of heparin mL^{-1} (Fisher Scientific, Waltham, MA). Pronephros samples were processed within 24h of extraction and the tissues were passed through 100μm mesh in 2mL of the modified L-15 medium. The samples were then placed on a 34%/51% Percoll (Fisher Scientific, Waltham, MA) density gradient and centrifuged at 500 x g for 30min. Cells were collected at the density interface and added to cold modified L-15 medium. The collected cells solutions were then centrifuged at 1000 x g for 10 min and the pellet was re-suspended in L-15 supplied with 0.1% FCS. Samples were adjusted to $2x10^7$ cells mL^{-1} using a hemocytometer, verified for viability using trypan blue, and $2x10^6$ cells were seeded in each well of a 96 well tissue culture plate. Cells were allowed to adhere for 2h at 18°C before being washed with fresh L-15 (non-supplemented). Adherent cells were incubated overnight in L-15 supplemented with 5% FCS prior to being used in the assays. Macrophage respiratory burst activity (RBA), derived from the pronephros was determined using spectrophotometric analysis of nitroblue tetrazolium (NBT) reduction via superoxide production. For the RBA reagent preparation, NBT was added at a concentration of 1mg mL^{-1} and 1μg mL^{-1} phorbyl 12-myristate 13-acetate was added to L-15 medium. 100μL of the RBA reagent was added to the wells and allowed to react for 30min. The wells were then emptied, the cells fixed with methanol, and the wells were washed twice with a 70% methanol rinse. 120μL of 2M potassium hydroxide (KOH) and 140μL of dimethyl sulfoxide (DMSO) were added

to each well and the plates were agitated and read in a microplate reader (BioTek Corp., Winooski, VT) at 620nm, using KOH/DMSO wells as blanks. OD_{620} readings were adjusted for blanks and were compared to the respective RBA.

Serum Lysozyme and Alternative Complement Activity

Serum lysozyme was detected using a turbidimetric assay with, lyophilized bacteria (*M. lysodeikticus*; Worthington Chemical, Lakewood, NJ) to determine inherent concentrations (Kim & Austin, 2006 ; Ellis, 1990). Briefly, 100 µl of serially diluted rainbow trout serum in 0.05M sodium phosphate buffer (SPB; pH 6.2) was added to 96-well plates. 100 µl of 0.4 mg ml^{-1} suspension of the bacteria in SPB and the plates were agitated to mix samples. The optical density (O.D.) was measured at 570nm at 0 and 15 min at 20°C. SPB was used alone as a microplate blank and replaced serum as a negative control. A unit of lysozyme activity was defined as the amount of serum causing a decrease in absorbance of 0.001 min^{-1}.

Alternative complement activity (ACH50) was determined using the hemolysis of sheep red blood cells (RBCs) according to previously described and published methods (Welker et al., 2014). Sheep RBCs (10% packed volume in Alsever's solution; Lampire Biological, Pipersville, PA) were washed 4 times (300 x g at 4°C) with cold PBS with 1% gelatin (PBS+; pH 7.2) and suspended in PBS+. The cell solution was adjusted with a hemocytometer to a final concentration of $5x10^7$ RBC mL^{-1}. In a 96-well round-bottom plate, 50 µl of rainbow trout sera were serially diluted in PBS+ (from 20% to 0.157% concentrations) and the volume diluted to 250µl. 50µl of the RBC solution was added to each well to bring the reaction volume to 300µl. Positive controls were used

for each sample to represent 100% hemolysis (250µL dH_2O and 50µL RBCs). Negative controls were used for each sample to represent spontaneous lysis (250µL PBS and 50µL RBCs). Samples were incubated at 21°C for 1hr with agitation and the reaction was stopped by placing the plates on ice for 3 minutes. Samples were centrifuged (800 x g for 10min at 4°C) and 250µL of supernatant was transferred to a 96-well flat bottom plate. The absorbance (410nm) was recorded using a plate reader (EPOCH model; BioTek. Winooski, VT). Complement hemolytic activity is expressed as ACH50 units mL^{-1}, where a ACH50 unit represents the volume of serum necessary to produce the lysis of 50% of sheep erythrocytes. The degree of hemolysis was estimated via the lysis curve for Y / (1 − Y) against the volume of serum added (µL). The Y value (percentage of hemolytic activity at each dilution relative to the positive and negative controls) was determined by the equation:

$$Y = 100 \times [\text{Abs(A)} - \text{Abs(B)}] / [\text{Abs (C)} - \text{Abs (B)}]$$

Where, Abs (A) = absorbance of the rainbow trout serum dilution; Abs (*B*)=absorbance of the negative control (spontaneous lysis); Abs (*C*) = absorbance of the positive control (100% RBC lysis).

Cytokine Analysis

Organs were extracted at the time of necropsy and preserved at -20°C in RNAlater (Life Technologies, Carlsbad, CA) to preserve sample quality. The spleen and head kidney samples were later homogenized by hand in a 1.7mL microcentrifuge tube using Trizol reagent (ThermoFisher Scientific, Waltham, MA), and purified RNA was extracted according to the manufacturer's directions. The total RNA concentrations and

A260/A280 ratios were determined using a Take3 microplate (BioTek Corporation, Winooski, VT) and EPOCH microplate reader (BioTek Corporation, Winooski, VT). The RNA was diluted to a concentration of 1μg μL^{-1} in RNase/DNase free, ultrapure water. 1μg of the adjusted total RNA was then used in a reverse transcription reaction to create complementary DNA (cDNA) for downstream processing, using the Maxima First Strand cDNA Synthesis Kit with dsDNase (ThermoFisher Scientific, Waltham, MA), according to the manufacturer's directions. The 20μl of cDNA was then stored at -20°C until required for use in the RT-PCR reactions. Previously published primer sets for rainbow trout cytokines and the β-actin housekeeping were constructed and obtained from a commercial supplier (IDT Technologies, Coralville, IA) in 25nmol quantities (Table 4; Bridle et al., 2006 ; Kim & Austin, 2006).

For RT-PCR setup, the previously transcribed cDNA was diluted 1:10 in RNase/DNase-free water and then 2μl (5ng) was added to a MicroAmp 96-well optical plate (Life Technologies, Carlsbad, CA) on ice. Primers were initially diluted to 100mM concentration in Tris-EDTA (TE) buffer and then 500nM concentrations of each primer was used within the reaction. 6μl of RNase/DNase free water, 1μl of forward primer, 1μl of reverse primer, and 10μl of PowerUp SYBR Green Master Mix (ThermoFisher Scientific, Waltham, MA) were added to the chilled wells, as per the product directions, for a total reaction volume of 20μl. The reaction plate was then spun at 1000 x g for 1 minute in an Eppendorf 5810R centrifuge (Rotor A-4-62 with plate holder; Eppendorf North America; Hauppauge, NY) and a MicroAmp optical plate cover (Life Technologies, Carlsbad, CA) was used to seal the plate.

The RT-PCR reactions were performed using an ABI 7900HT (Applied Biosystems, Foster City, CA) and the associated SDS 2.4 software. Thermal profiles from Kim & Austin (2006) were used for the primer pairs, with some modifications. The RT-PCR reaction program was as follows: initial denaturation at 95°C for 3min, followed by 40 cycles of denaturation (95°C for 20s), annealing (57°C for 20s for β-actin; 61°C for 20s with IL1β and TNF-α), and elongation 72°C for 30s. Lastly, a dissociation curve was then performed following the amplification phase to verify product homogeneity and the lack of primer-dimers. RT-PCR data was then analyzed using the Livak equation ($2^{-\Delta\Delta Ct}$), as previously performed in rainbow trout cytokine analysis (Chettri et al., 2011). The β-actin reference gene was used to normalize the data and treatment diets were compared to the fishmeal control to elucidate differences in cytokine gene expression at the two time points.

Statistical Analysis

JMP 12 (SAS Corporation, Cary, NC) and Program R (R Core Development Team, Vienna, Austria) were used to perform data analysis of trial results. Analysis of variance (ANOVA) was used to compare immune metrics, while Tukey's HSD was used for multiple comparisons, when applicable. Nonparametric data were analyzed with the Kruskal-Wallis one-way analysis by rank and Mann-Whitney U tests for comparisons. Results from statistical analyses were deemed significant at $\alpha=0.05$.

Results

Macrophage RBA was found to be significantly different at day 46 (P=0.004) and post-challenge 10 (P<0.001), while day 60 levels did not vary across dietary treatments (P=0.427; Figure 1). At day 46, the FM control diet had a significantly lower RBA level (OD_{620}= 0.373) than the EXOP (P=0.004) and β-glucans (P=0.003) supplemented diets. Post-10 day challenge results indicate a decrease in RBA across all treatment diets, in comparison to the FM control. The FM control demonstrated a statistically greater RBA than the β-glucans (P=0.012), BSBM-1 (P<0.001), and BSBM-2 (P<0.001).

Lysozyme levels were found to have significant differences among diets at day 46 (P<0.001) and day 60 (P<0.001), but no significant differences were found as lysozyme levels decreased across all treatments following the *F. psychrophilum* challenge (P=0.695; Figure 2). The β-glucans treatments displayed the lowest serum lysozyme at day 46 (213.0±2.8 U mL^{-1}) and day 60 (260.0±2.3 U mL^{-1}). Although no significant treatment differences were found following the bacterial challenge, the BSBM-1 diet (194.2±10.6 U mL^{-1}) had the greatest serum lysozyme levels of the treatment groups. The analysis of alternative complement activity in the sera did not yield significant treatment differences at day 46 (P=0.236), day 60 (P=0.288), and post-challenge 10 (P=0.078).

The post-challenge period cumulative mortality of all dietary treatments is summarize in Figure 3. Although not significantly different (P=0.3368), mortality at post-challenge day 10 were found to be lower in diets BSBM-2 (47.91±6.24%), BSBM with 1% EXOP (49.55±8.28%), and BSBM with 0.1% β-glucans (40.47±7.15%), in comparison to the FM control diet (59.45±5.44%) and BSBM-1 treatment (58.93±9.13%). No significant findings were determined in the histological review of spleen, kidney, and gill tissues and *F. psychrophilum* was not detected within the tissue

slides. Perisplenitis or mild gill inflammation were apparent in some slides, but no treatment-related differences could be discerned.

No differences were found in ACH50 values across treatments at day 46 (P=0.236), day 60 (P=0.288), and post-challenge (P=0.078; Figure 4). Although not significantly different, the post challenge BSBM1 (27.57±6.00 U/mL) and BSBM2 (40.34±4.63 U/mL)groups had the lowest ACH50 values.

IL-1β and TNF-α showed some relative variation from the fishmeal control in kidney samples at day 60 and spleen samples at post-challenge 10 (Figures 5-8). IL-1β levels (P=0.942) and TNF-α (P=0.234) expression levels appeared be decreased in the treatment diets in the head kidney tissues sampled at day 60, with the exception of a slight increase in TNF-α within the BSBM-2 group (0.3 fold increase). No differences were reported among post-challenge treatment groups for IL-1β (P=0.999) and TNF-α (P=0.6195) expression. The post-challenge splenic cytokine gene expression changes showed an increase in IL-1β in the BSBM-2 group (1.7 fold increase), while the TNF-α expression levels were all reduced in comparison to the FM control.

Discussion

New approaches and research directions to *F. psychrophilum* infectivity in salmonids have recently come to light in salmonid aquaculture. Breeding programs and resistant strains have been selected for commercial application and resistance mechanics are currently being investigated (Weber et al., 2013). Alternatively, several research projects have focused on *F. psychrophilum* phage therapy to attempt to curb the ubiquitous strains in hatchery settings (Madsen et al., 2013; Castillo et al., 2012 ;

Stenholm et al., 2008). The current diet-based research effort attempted to investigate several components of the relatively unknown innate immune response in rainbow trout, when exposed to *F. psychrophilum*.

The incorporation of β-glucans and EXOP to the BSBM diet elevated the macrophage RBA at 46 days into the feeding trial, while the base BSBM products also showed some increase, although not statistically significant, from the FM control. This boost to overall macrophage activity with dietary β-glucans supplementation has been widely observed in fish species (Guzman-Villanueva et al., 2014 ; Chang et al., 2013). As there were no marked increases in the treatment diets post-challenge, other macrophage attributes, such as phagocytosis index may be helpful in determining *F. psychrophilum* infectivity at a cellular level. Decostere et al (2001) found that the *in vivo* phagocytic capacity of splenic macrophages changed with the age class of the fish, and that younger rainbow trout had more prevalent intracellular *F. psychrophilum* concentrations (Decostere et al., 2001). Furthermore, *F. psychrophilum* has the ability to overcome the respiratory burst of macrophages and can be cytotoxic to the cells when it is phagocytized, potentially lowering the innate immune response of the trout to bacterial infection (Nematollahi, 2003). Thus, a more pronounced respiratory burst activity going into challenge trials may be able to compensate for some of the inherent bacterial evasion tactics. With the incorporation of dietary immunostimulants, the administration regime may be a key component of maintaining an enhanced and effective cellular innate immune response (Bricknell & Dalmo, 2005).

A decrease in serum lysozyme levels from the pre-challenge sampling values was apparent among all dietary treatment groups following the *F. psychrophilum* challenge.

These results are similar to previous findings that have characterized the immunosuppressive effects of *F. psychrophilum* on innate immune parameters, including lysozyme and total immunoglobulin levels (Siwicki et al., 2004). Immunostimulants are known to provide an increase to lysozyme production in fish, so the administration dynamics may be of importance to retain elevated the responsiveness of innate immune parameters (Maqsood, 2011). Further, it has been found that *F. psychrophilum* can resist serum complement activity, thus potentially providing an explanation for some of the decreased ACH50 values in the post-challenge period, although dietary effects were not significant (Nagai & Nakai, 2011; Wiklund & Dalsgaard, 2002).

Modulations to the cytokine response among diets in the spleen tissue extracted from challenged fish did not exhibit large-scale differences in expression ratios. It has been widely reported that cytokine levels may fluctuate as a result of dietary immunostimulants and probiotics (LaPatra et al., 2014; Panigrahi et al., 2007). IL-1β levels have been found to be overly expressed in gills, spleen and kidney of rainbow trout during challenge with *F. psychrophilum* (Orieux et al., 2013). Further, a previous study that evaluated environmental pathogen interactions in wild trout reported that TNF-α has also found to be associated (up-regulated) with the presence of *F. psychrophilum* (Wellband & Heath, 2013). As the current study investigated changes in gene expression in relation to a dietary control, the inclusion of an unchallenged control group may better elucidate some of the cytokine expression modulations associated with disease onset. Further investigation of gene expression changes to both inflammatory cytokines and genes that modulate immune parameters, such as C3, may be of importance to better

understanding the infectivity mechanics of *F. psychrophilum* infection (Langevin et al., 2012).

As treatment regiments and management practices are implemented to protect *F. psychrophilum* outbreaks, the current study suggests some dietary-related to changes to survival that warrant further investigation. While a commercial vaccine is closer to production, these immunostimulants and plant-based proteins may provide better protection for salmonids during seasonal periods where *F. psychrophilum* infectivity is most compromising. Furthermore, these diet-related health enhancements may also reduce the need for implementing antibiotic treatments for *F. psychrophilum* outbreaks (Wagner et al., 2012).

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Tables

Table 1. Ingredient proximates of experimental protein sources (dmb) used in the feeding trial. FM (fishmeal), BSBM (bioprocessed soybean meal).

Proximates	FM	BSBM-1	BSBM-2
Crude Protein (g $100g^{-1}$)	71.25	65.44	63.14
Crude Lipid (g $100g^{-1}$)	9.24	1.20	0.93
Crude fiber (g $100g^{-1}$)	0.10	6.35	5.77
Ash (g $100g^{-1}$)	20.38	4.96	4.52
Moisture (%)	8.01	8.45	11.09

Table 2. Proximate analysis (dmb) of formulated trial diets used in the rainbow trout feeding trial. FM (fishmeal), BSBM (bioprocessed soybean meal).

Proximates	FM	BSBM-1	BSBM-2
Crude Protein (g $100g^{-1}$)	40.33	39.69	38.39
Crude Lipid (g $100g^{-1}$)	19.82	18.00	19.58
Crude fiber (g $100g^{-1}$)	0.91	2.36	2.19
Ash (g $100g^{-1}$)	7.97	5.72	5.50
Moisture (%)	5.64	4.47	3.99

Table 3. Experimental diet formulations and analyzed proximate composition of trial diets. FM (fishmeal), BSBM (bioprocessed soybean meal), βG (beta glucans), EXOP (exo-polysaccharide).

Constituent	FM	BSBM-1	BSBM-1 +βG	BSBM-1 +EXOP	BSBM-2
Fishmeal[a]	20.0	5.00	5.00	5.00	5.00
BSBM-1	-	19.47	19.47	19.47	-
BSBM-2	-	-	-	-	19.47
Dextrose	1.50	1.03	0.93	0.03	1.03
Corn gluten[b]	17.50	15.50	15.50	15.50	15.50
Wheat flour[c]	16.53	16.53	16.53	16.53	16.53
Wheat gluten[c]	12.00	10.00	10.00	10.00	10.00
Rice bran[b]	7.00	7.00	7.00	7.00	7.00
Vitamin premix[d]	1.00	1.00	1.00	1.00	1.00
Mineral premix[e]	0.10	0.10	0.10	0.10	0.10
Micro-ingredients	9.17	9.17	9.17	9.17	9.17
β-glucans	-	-	0.10	-	-
EXOP	-	-	-	1.00	-
Brewers yeast[f]	0.20	0.20	0.20	0.20	0.20
Fish Oil[g]	15.00	15.00	15.00	15.00	15.00
Total	100.0	100.00	100.00	100.00	100.00
Protein	42.14	42.47	42.47	42.0	41.96
Lipid	18.21	17.51	17.51	17.51	17.37
Fiber	1.21	2.37	2.37	2.37	1.15
Ash	10.69	7.49	7.49	7.49	6.58

[a] Special Select, Omega Protein, Houston, TX;[b] Ag First Cooperative, Brookings, SD; [c] Bob's Red Mill Natural Foods, Milwaukie, OR; [c] USB Corporation, Cleveland, OH; [d] ARS 702 premix, Nelson and Sons, Murray, UT; [e] SS #3 trace mix, Nelson and Sons, Murray, UT; [f] Diamond V, Cedar Rapids, IA; [g] Virginia Prime Gold, Omega Protein, Houston, TX.

Table 4. Primer pairs for cytokine expression levels in head kidney and spleen samples extracted at day 60 and post-challenge 10. Rainbow trout primers were previously published by Kim & Austin (2006) and Bridle et al. (2006), based on rainbow trout cytokine sequences in GenBank. Bp=base pairs, F=Forward primer sequence, R=reverse primer sequence.

Gene of Interest	Product Size (bp)	Oligonucleotide Sequence (5'-3')	Accession No.
β-actin	186	F: GGACTTTGAGCAGGAGATGG R: ATGATGGAGTTGTAGGTGGTCT	AJ438158
IL-1β	181	F: ACCGAGTTCAAGGACAAGGA R: CATTCATCAGGACCCAGCAC	AJ223954
TNF-α	208	F: CAAGAGTTTGAACCTCATTCAG R: GCTGCTGCCGCACATAAAG	-

Figures

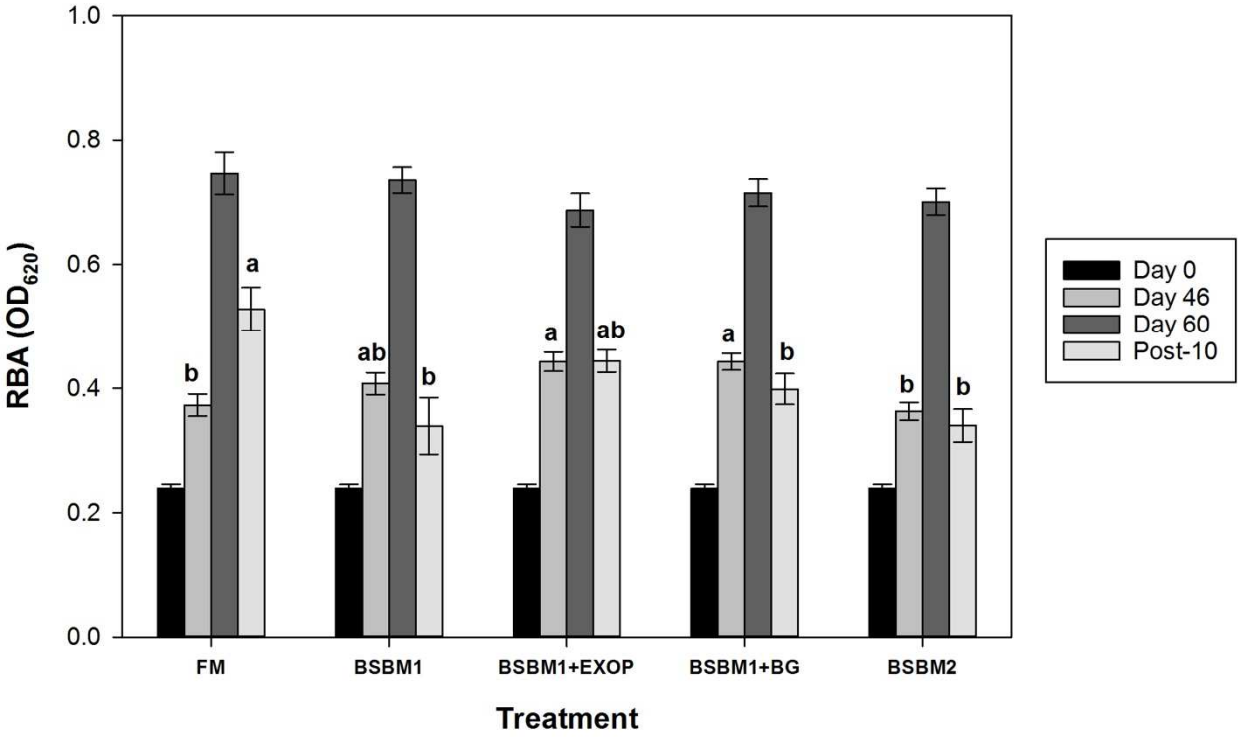

Figure 1. Respiratory burst activity (RBA) of head kidney macrophages sampled at days 0, 46, 60, and post-challenge 10. Different letters denote significant differences between dietary treatments for each group at each sampling date. FM (fishmeal), MSBM (modified soybean meal), EXOP (exo-polysaccharide), βG (beta glucans).

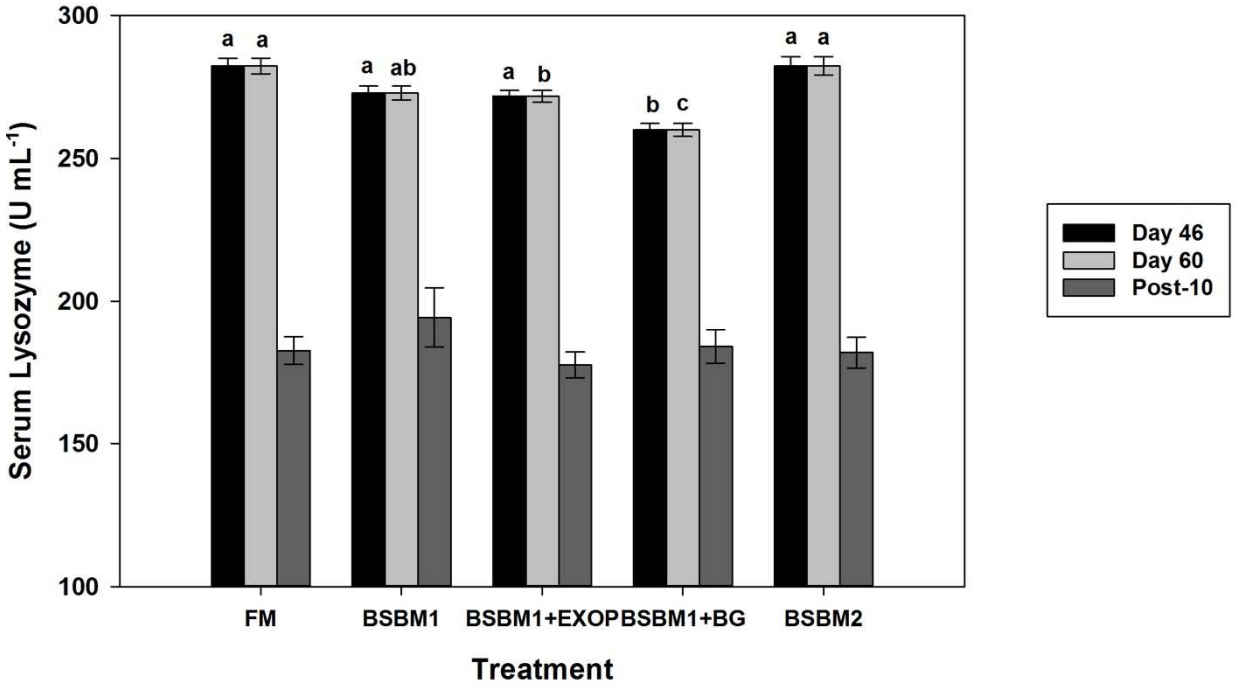

Figure 2. Serum lysozyme content sampled at days 0, 46, 60, and post-challenge 10. Different letters denote significant differences between dietary treatments for each group at each sampling date. FM (fishmeal), MSBM (modified soybean meal), EXOP (exo-polysaccharide), βG (beta glucans).

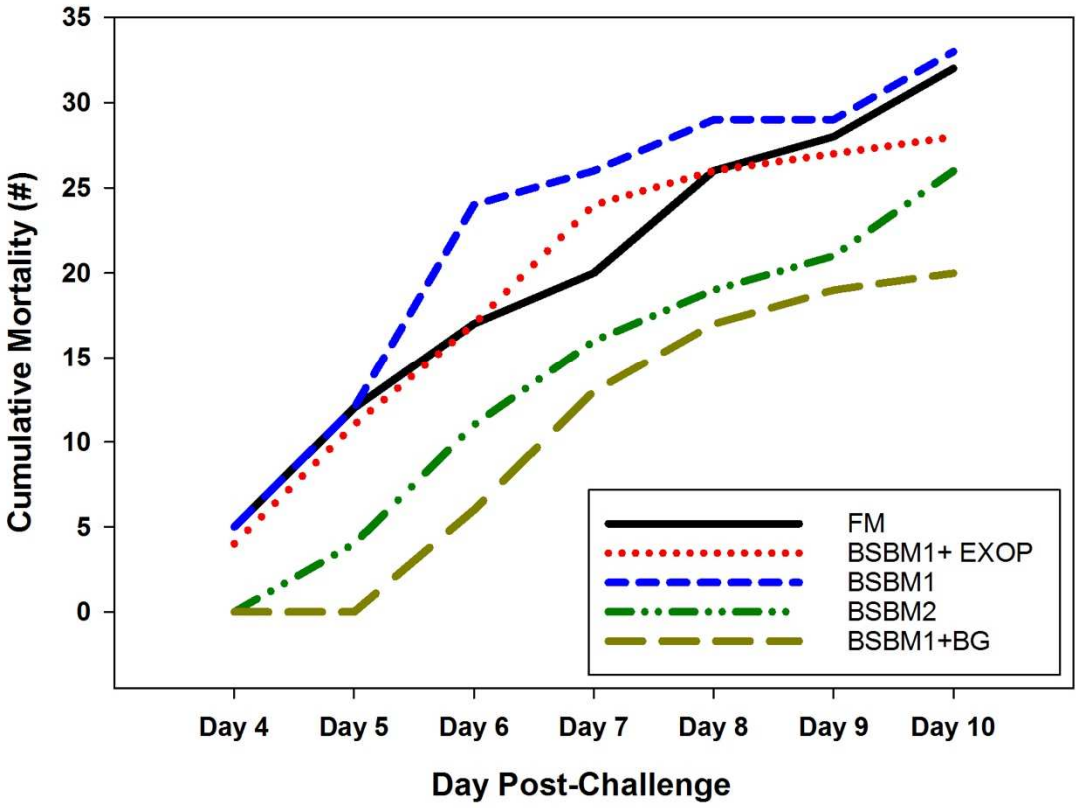

Figure 3. Cumulative mortality curve from days 1-10 post-challenge with *Flavobacterium psychrophilum* strain 17830. FM (fishmeal), MSBM (modified soybean meal), EXOP (exo-polysaccharide), βG (beta glucans).

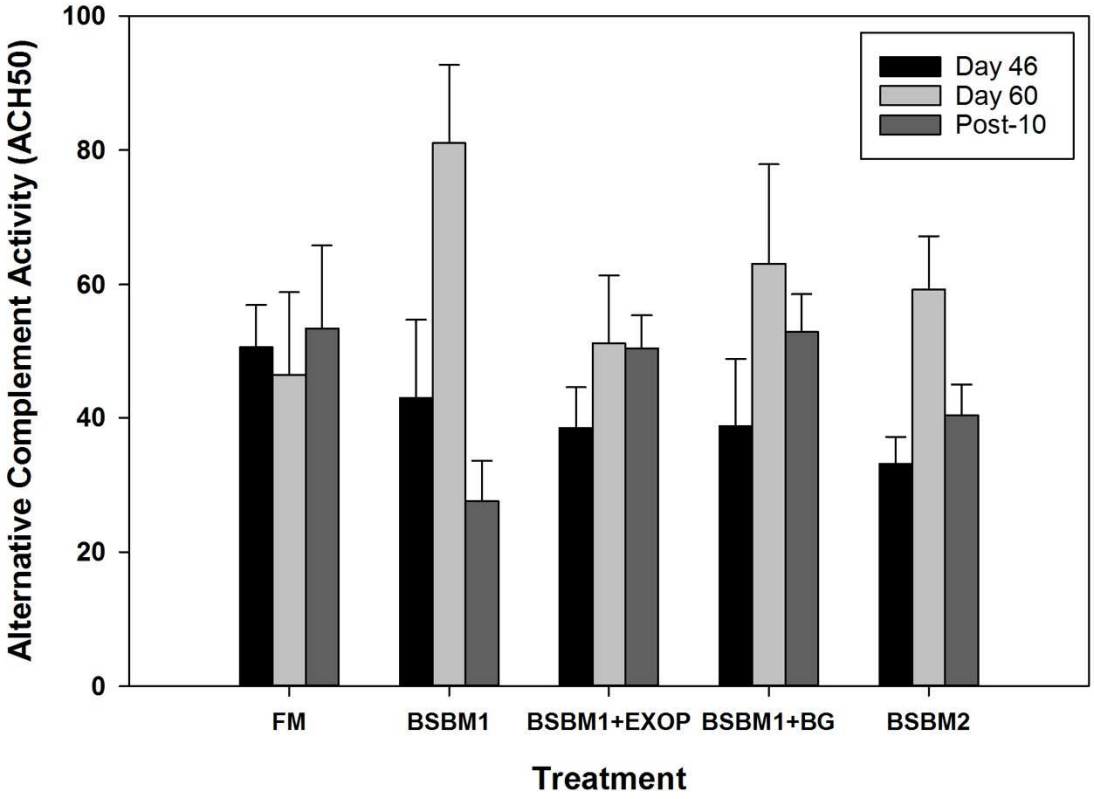

Figure 4. Experiment alternative complement activity (ACH50) for days 46, 60, and post-challenge 10. FM (fishmeal), MSBM (modified soybean meal), EXOP (exo-polysaccharide), βG (beta glucans).

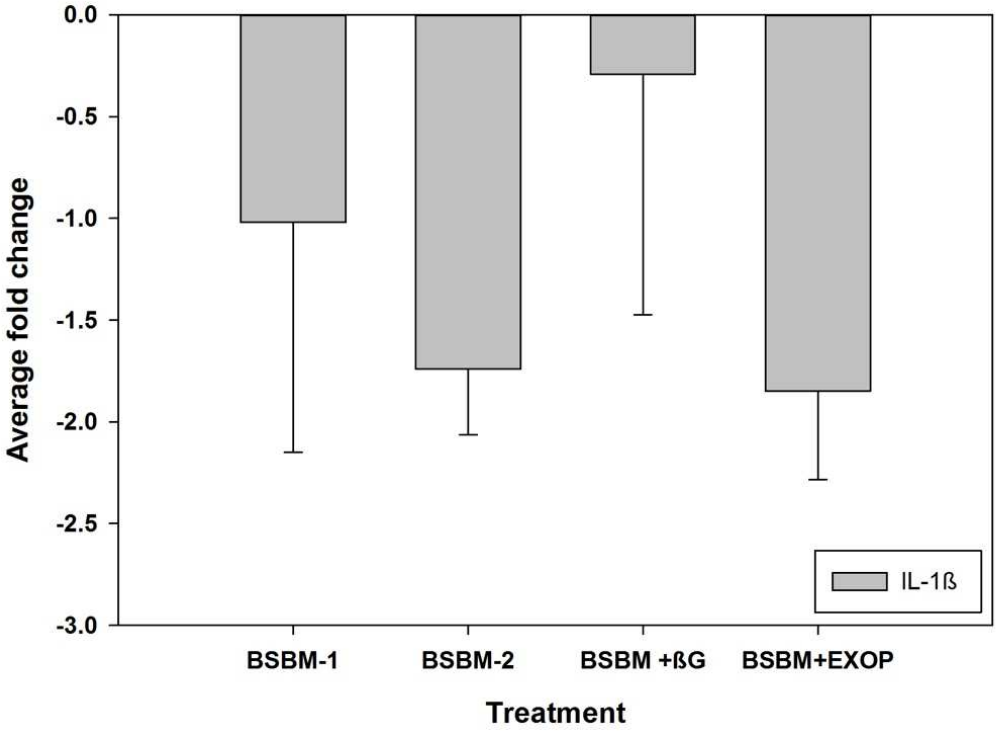

Figure 5. Relative expression levels of day 60 head kidney IL-1β, in comparison to the FM reference diet. FM (fishmeal), MSBM (modified soybean meal), EXOP (exo-polysaccharide), βG (beta glucans).

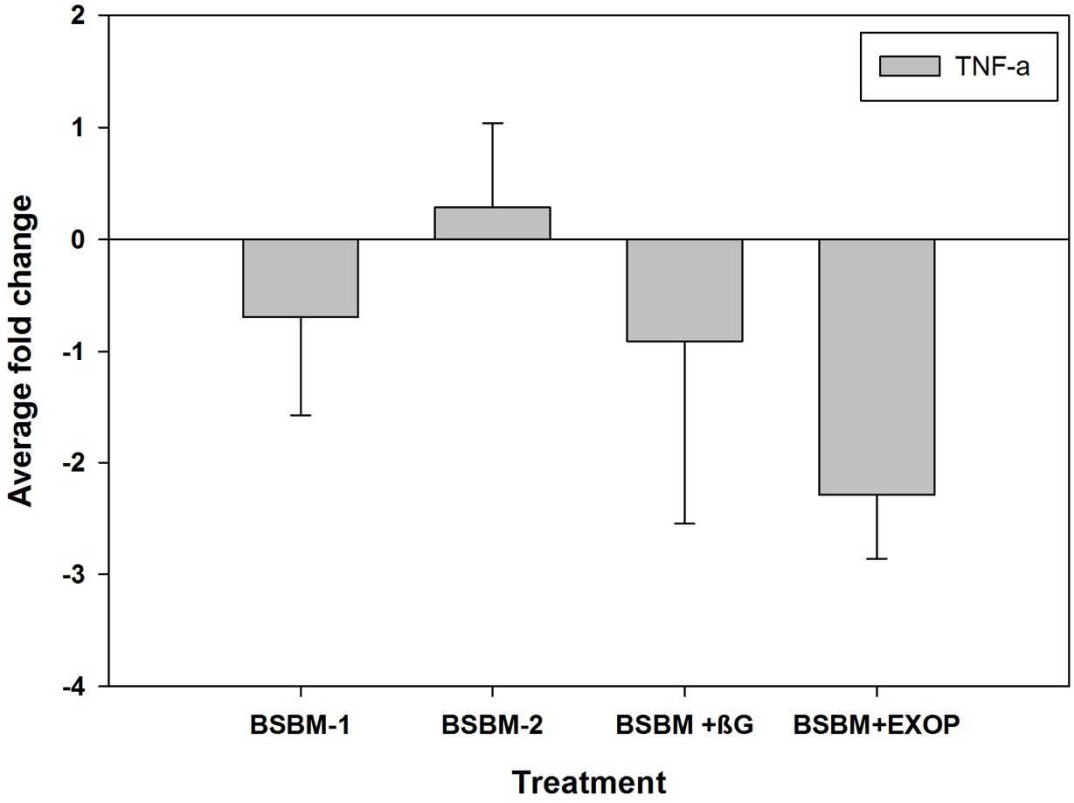

Figure 6. Relative expression levels of day 60 head kidney TNFα, in comparison to the FM reference diet. FM (fishmeal), MSBM (modified soybean meal), EXOP (exo-polysaccharide), βG (beta glucans).

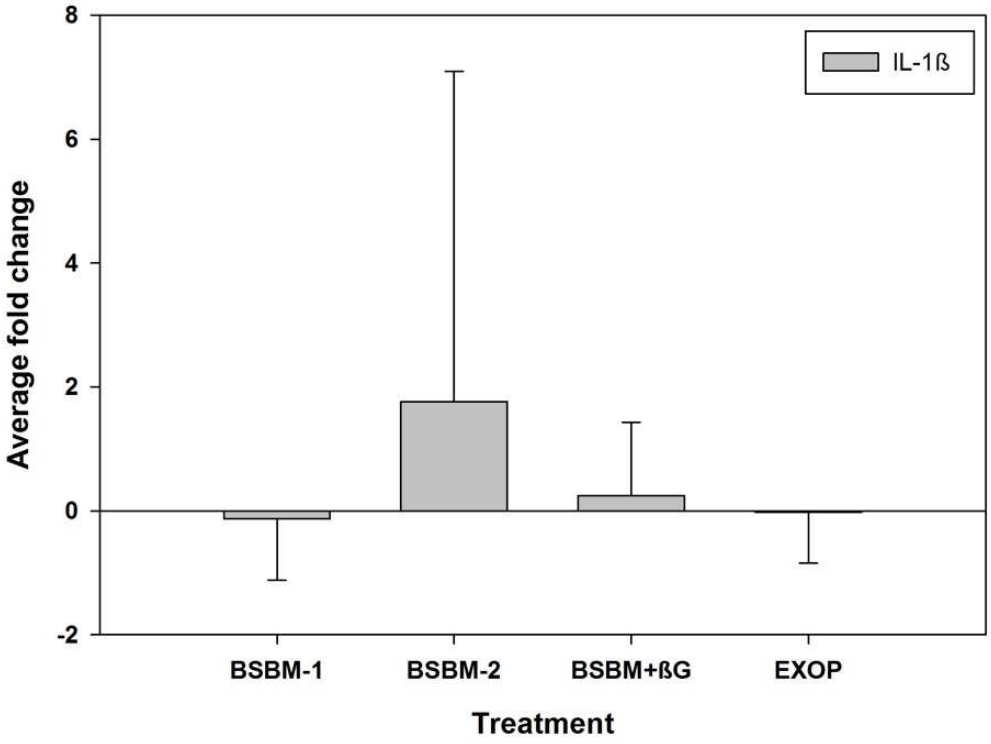

Figure 7. Relative expression levels of post-challenge 10 spleen IL-1β in comparison to the FM reference diet. FM (fishmeal), MSBM (modified soybean meal), EXOP (exo-polysaccharide), βG (beta glucans).

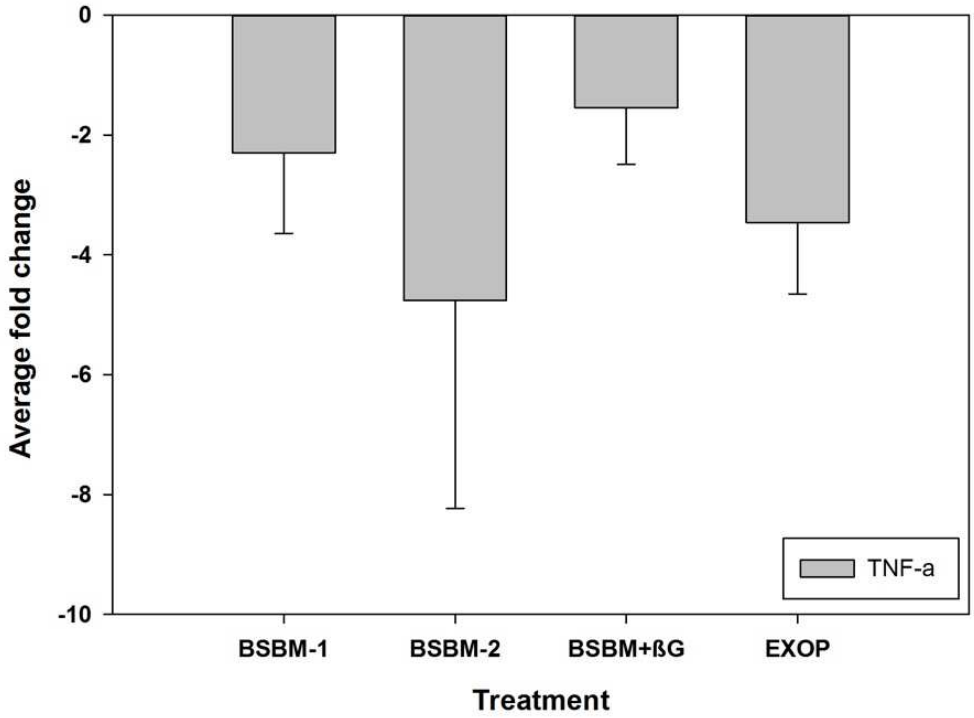

Figure 8. Relative expression levels of post-challenge 10 spleen TNF-α, in comparison to the FM reference diet. FM (fishmeal), MSBM (modified soybean meal), EXOP (exo-polysaccharide), βG (beta glucans).

CHAPTER 5. INTESTINAL IMMUNOLOGY AND GUT MICROBIOTA CHANGES IN RAINBOW TROUT *Oncorhynchus mykiss* FED BIOPROCESSED SOYBEAN DIETS

Abstract

Processed soybean meal ingredients have become an emerging plant-based protein used in aquafeed formulations and shown to influence gastrointestinal health and microflora in cultured species. Two 60-day feeding trials with juvenile rainbow trout (*Oncorhynchus mykiss*) were conducted to compare the effects of fishmeal (FMC), defatted soybean meal (SBM), bioprocessed soybean meal (BSBM), and commercial soy protein concentrate (CSPC) ingredients on intestinal histology, innate immunity, and microbiota profiles. Results indicated no significant differences in intestinal immunoglobulin concentrations ($P=0.41$) or gut leukocyte phagocytosis at day 15 samplings ($P=0.41$). Intestinal lysozyme content was found to be highest at day 60 in the BSBM treatment group ($P<0.01$). No significant differences across treatments were observed in histological profiles at day 0 ($P=0.75$), day 15 ($P=0.08$), and day 60 ($P=0.22$). ANOSIM analysis for microflora diversity showed differences among all treatment groups, with the exception of the high and low levels of BSBM ($P=0.40$). There were also differences observed in the alpha diversity ratios, with significant differences in gut communities among dietary groups as demonstrated by Chao-1 ($P<0.01$) and Shannon indices ($P=0.02$). The incorporation of processed soy-based proteins alters the microbial community composition within the rainbow trout gastrointestinal tract (GIT), and modulates lysozyme concentrations within the distal gastrointestinal tissues.

Introduction

Alternative plant-based aquafeeds have been of recent interest for commercial fish producers and aquaculture researchers, as the industry seeks high-protein, cost-effective replacements for marine-derived fishmeal (Hardy, 2010). Of these plant proteins, soybean meal (SBM) has emerged as a candidate for fishmeal replacement in many feed formulations, due to a relatively high protein level and an amino acid profile that approaches the nutritional requirements of many cultured species (Refstie et al., 2000). To further improve soy ingredient profiles, processing efforts have yielded increased protein levels, decreased levels of antinutritional factors (ANFs), and enhanced digestibility profiles (Zhou et al., 2011 ; Barnes et al., 2012 ; Chen, 2013). SBM has been found to modulate many aspects of gastrointestinal tract (GIT) health within fish species, including the histological composition, immune status, and the overall GIT microbiota (Ringø et al., 2006 ; Merrifield, 2011 ; Barnes et al., 2015 ; Ringø et al., 2016). High inclusion rates of soybean meal have been found to induce intestinal enteritis, increase GIT inflammatory immune responses, and influence GIT microbe composition (Rumsey et al., 1994 ; Heikkinen et al., 2006 ; Venold et al., 2012). These responses are often species-specific, traditionally impacting salmonid GITs while appearing relatively unobtrusive in other species, such as cod (*Gadus morhua*) and cobia (*Rachycentron canadum*) (Romarheim et al., 2008 ; Refstie et al., 2006). In comparison to an abundance of fish GIT research conducted using unprocessed SBM inclusions, there have been relatively few studies that compare these metrics in fish fed upgraded soy products such as bioprocessed soybean meal (BSBM) or commercial soy protein concentrates (CSPCs). Furthermore, inclusions of these upgraded soy protein sources

appear to both positively and negatively impact fish GIT health and microbe community structure, necessitating the need for further research with novel soy products (Navarrete et al., 2013 ; Green et al., 2013). To evaluate the effect of processed SBM-based diets on overall gut health and microbiota composition, controlled feeding trials were conducted to determine morphological and immunological changes in rainbow trout (*Oncorhynchus mykiss*).

Materials and Methodology

Experiment A: Histological and immunological sampling

The first 60-day experiment took place in biosecure laboratory (BSL). One hundred and eighty juvenile rainbow trout (13.70±0.18 g) were randomly stocked into 12, 38L tanks. Three diets were formulated and included fishmeal, modified bioprocessed soybean meal (MSBM), and commercial soybean concentrate (CSPC) as main protein sources (Table 1). Each dietary treatment was allocated to 4 replicate tanks for the study duration. Following an acclimation period of 7 days, 4 fish randomly selected fish per tanks were removed and measured for mean (±SE) total length (11.40±.0.94 cm) and weight (17.21±0.51 g). The study tanks received replacement well water and the recirculating aquaculture system (RAS) was maintained at optimal conditions for rainbow trout culture (15.4°C, 7.02 mg L^{-1} dissolved oxygen, pH 7.65). Fish were fed by hand twice and daily feed rations were dispensed at 4% body weight, adjusted every 15 days (day 15, day 30, and day 45) based on the specific growth rate from the day 0 to day 15 interval. No palatability or feeding issues were detected across any of the dietary

treatments. Fish were found to be healthy during the entire trial and no mortalities were noted over the 60 days.

Three diets were formulated on an isonitrogenous for the short-term feeding trial (Table 2). A fishmeal control diet (FMC) contained 38.1% menhaden fishmeal (Special Select, Omega Prime, Houston, Texas) as the primary protein source, while the other two diets contained only 9.97% menhaden fishmeal, providing approximately 73% menhaden fishmeal replacement. A proprietary microbial bioprocess (Prairie AquaTech, Brookings, South Dakota) was used to manufacture the BSBM protein for this study while the CSPC was obtained from a commercial source (Soycomil P, Archer Daniels Midland, Chicago, Illinois).

Diets were prepared by grinding the large particle ingredients with a Fitzpatrick Comminutator (Elmhurst, Illinois) with 1.27 mm screen prior to dry blending. Dry feedstuffs were blended in a ribbon mixer (Patterson Equipment, Toronto, Ontario) and diets were extruded using a Extrutech E325 single-screw extruder. Extruded pellets were dried with a conveyor oven drier (Colorado Mill Equipment, Canon City, Colorado), sifted with a Rotex screener (Rotex Inc., Cincinnati, Ohio) and then lipid coated using a Phlauer vacuum fat coater (A&J Mixing, Oakville, Ontario). Diets were cooled to room temperature, bagged, and stored at 4°C pending use.

Experimental diets were dispensed to tanks beginning on day 1. Four randomly selected fish per tank were sampled for immunological assays on days 0, 15, and 60. Length and weight to the nearest 0.01g or 0.1mm, respectively, were recorded for each individual fish to calculate Fulton's condition factor (K-value) as follows:

$$\mathrm{K} = \frac{\text{final weight (g)}}{\text{total length (mm)}^{3}} \times 100{,}000$$

From two randomly selected fish per tank, approximately 2-cm distal intestine sections were excised from each fish and fixed in 10% formalin for histological analyses, or stored in 1xPBS (pH 7.2) for intestinal homogenate preparation.

Experiment B: Gut microbiota sampling

The second 60-day feeding trial used 420 juvenile rainbow trout (42.30±0.26 g) randomly stocked into 30, 114L tanks connected in parallel to an RAS. Each dietary treatment was allocated to 5 replicate tanks for the study duration, with an initial density of 14 fish per tank. Six diets were formulated and included a fishmeal control, a defatted soybean meal control (SBM), two inclusion levels of BSBM, and a CSPC as the treatment protein sources (Table 1); diet processing methods were similar to the first experiment (Table 3).

Following an acclimation period of 7 days, diets were administered for a period of 60 days. The study tanks received replacement water and the RAS was maintained at suitable conditions for rainbow trout culture (15.26°C, 6.99 mg L^{-1} dissolved oxygen, pH 8.32). Fish were fed by hand twice daily and feed rations were 2% body weight per day. No palatability or feeding issues were detected for any of the dietary treatments, including the SBM negative control. Fish were found to be healthy during the entire trial, with the exception of 10 mortalities noted over the 60 days, due to escapes (1), undiagnosed illness (8 total; 4 from the SBM treatment, 2 from the high inclusion BSBM treatment, and 2 from the FMC treatment), or insufficient flow rate (1) in one instance.

The size distribution of the randomly selected day 60 fish was as follows: FMC (146.8±9.5g), SBM (163.2±7.6g), CSPC (163.2±16.1g), high inclusion BSBM

(153.9±15.0g), and low-level BSBM (159.4±13.1g). For gut microbiota analyses, distal intestine fecal samples were collected at random from 5 individuals per treatment and flash-frozen in liquid nitrogen. Samples were then shipped on dry ice to a commercial lab (Research and Testing Laboratories, Lubbock, Texas) for DNA extraction and 16S rRNA sequencing.

Intestinal leukocyte isolation and phagocytosis assay

Gastrointestinal leukocytes were isolated using previously reported methods by Clerton et al. (2001), with minor modifications to the phagocytosis protocol. Gut leukocytes were isolated by incubating pieces of distal intestine in Hank's balanced salt solution (HBSS) supplemented with 0.370 mg mL^{-1} ediaminetetraacetic acid (EDTA) and 0.145 mg mL^{-1} dithiothreitol (DTT) for 30 min at 20°C in a refrigerated incubator. Intestinal tissue was rinsed in Dutch modified RPMI-1640 cell culture medium (RPMI; Fisher Scientific, Waltham, Massachusetts) to remove residual DTT. The tissue was then transferred to a 15 mL centrifuge tube containing Dutch modified RPMI-1640 medium (RPMI) with 0.35 mg mL^{-1} collagenase (Type II; Worthington Biochemical) and 5% fetal calf serum (FCS) for 30 min at 25°C. The supernatant was pushed through a 100µm cell strainer and centrifuged at 400 x *g* for 10 min. Cell viability was determined by the trypan blue exclusion method on a hemocytometer and the cell suspension adjusted to a concentration of $2x10^7$ RPMI.. 100 µl of the leukocyte suspension ($2x10^6$ intestinal cells in RPMI supplemented with 5% FCS) was plated in 12-well tissue culture plates. 9µL of 0.92µm green fluorescent beads (Fisher Scientific, Waltham, Massachusetts), approximately 1:10, were added to the treatment wells. Plates were incubated at 20°C for

4h prior to rinsing wells twice with 1xPBS (pH 7.2). The wells were then aspirated and 500µl of cold paraformaldehyde was added to each well.

Cells were imaged on an Olympus IX70 inverted compound microscope, equipped with epi-fluorescence (Olympus Corporation, Center Valley, Pennsylvania). Images were taken at 200X with and without the green fluorescent filter, to visualize cellular uptake of the FITC-labelled microbeads. A minimum of 100 cells were visualized in each field and the percentage of cells containing beads were considered to have been active in phagocytosis. The particle analyzer function in ImageJ v150 (NIH, Bethesda, Maryland) was used to count cells and cells containing fluorescence beads and the values were compared to yield of the percentage of cells that phagocytized the particulates.

Intestinal lysozyme content

Lysozyme levels were determined from distal homogenate (1g of intestine in 4 mL of distilled water) that was homogenized for 30s in a Polytron PT 2500E (Kinematic Inc.; Bohemia, New York). Previously reported methods for lysozyme analysis were used, with modifications for the intestinal homogenate (Kim & Austin, 2006 ; Bakke-McKellep, 2007). This turbidimetric assay used a 96-well microplate with 25ul of sera with the *Micrococcus lysodeikticus* suspension (0.2 mg/mL) in 0.05M sodium phosphate buffer. Absorbance is measured at 570 nm at 0 and 15min in an EPOCH microplate reader (BioTek, Winooski, Vermont) and a unit of lysozyme activity is defined as a change in 0.001 absorbance over the time interval. Results were expressed as lysozyme content in U/mL of tissue homogenate.

Histological evaluation of distal intestine

Intestinal tissues were sectioned and processed with hematoxylin and eosin staining. A total of 72 slides were processed, 24 from each of the three sampling time points. Slides were examined using 100x magnification (Nikon E-200, Melville, New York) and the segments were evaluated using previously reported methodology (Barnes, 2014; Table 4). Two separate reviewers independently analyzed all slides at random and assigned a ranking to each slide, based on the overall intestinal appearance and composition. Slides with a ranked difference of ±4 were re-examined by both reviewers for confirmation. Assessed ranks were compiled and averaged for overall gut scoring.

16S rRNA gut microflora profiling

DNA from the trout fecal material was extracted and sequenced using established protocols. Briefly, extracted fecal DNA was subjected to PCR amplification with primer pair 28F and 388R, and then sequenced with the Illumina MiSeq platform (Illumina, Inc., San Diego, California), with a 10K minimum. The post-sequencing pipeline incorporated de-noising, chimera checking, and quality checking. The sequences were then clustered into operational taxonomic units (OTUs), selected using UPARSE (Edgar, 2013), and mapped to an OTU data table using the USEARCH global alignment algorithm (Edgar, 2010). The multiple sequence aligner MUSCLE (Edgar, 2004) was used with a maximum of 2 iterations in order to perform OTU data alignment and passed into FastTree to infer approximate maximum-likelihood phylogenetic trees (Price et al., 2009). The USEARCH global alignment program was applied for taxonomic identification and diversity data

were trimmed using confidence values based on the amount of matching taxa to the total number of database-established taxa (Edgar 2010). The cleaned data was then analyzed for microbial diversity using PAST3 software (Hammer et al., 2001). Alpha diversity was compared using Chao-1 and the Shannon (H) indices. Similarities between treatment diet groups were analyzed using an analysis of similarities (ANOSIM) with pairwise comparisons. Further, similarity percentage analysis (SIMPER) was done to provide the Bray-Curtis measure of similarity.

Intestinal immunoglobulin levels

Total immunoglobulin (Ig) was determined using methods adapted for intestinal homogenate (Siwicki et al., 1994). Briefly, isolated homogenate (as listed in the lysozyme assay methodology) was further diluted to 1:4 in a 96-well plate with 0.85% saline solution and bicinchoninic acid (BCA) protein assays (Pierce Biotechnology, Rockford, IL) were used to read total protein in the homogenate. 100µl of homogenate was added to 100µl of a 12% polyethylene glycol, incubated for 2h with shaking, and centrifuged at 1000 x g to collect the supernatant. The supernatant was then processed using the BCA protein kit and the difference in absorbance calculated from the total protein calculation to derive total immunoglobulin content within the intestinal homogenate.

Statistical analysis

Other than data analyses completed for microflora profiles, analyses were conducted with JMP, Version 12 (SAS Institute Inc., Cary, North Carolina). All data

were first tested for normality using the Shapiro-Wilk test and equivalent variances using Levene's test. Analysis of Variance (ANOVA) was used to compare treatment results and significant outcomes were tested post hoc using Tukey's HSD for multiple comparisons. Nonparametric and ranked data were analyzed using the Kruskal-Wallis rank test and post hoc multiple comparisons using the Steel-Dwass method. An *a priori* alpha value of α=0.05 was used for all statistical analyses.

Results

Histological evaluation, immune parameters, and condition factor

Experiment A results showed no statistically significant differences across treatments were observed in histological profiles at day 0 (P=0.75), day 15 (P=0.08), and day 60 (P=0.22; Figure 1). Within each treatment, more variation between sampling intervals was noted compared to the FM reference diet. The CSPC treatment group showed the highest score (most negative intestinal profile) at day 15, but scores decreased over a longer time period.

Analysis of intestinal leukocyte phagocytosis at day 15 indicated no significant differences among treatments (P=0.41) (Table 5). No significant differences were also found in total Ig content at day 15 (P=0.41) and day 60 (P=0.20; Table 5). At day 15, there was no significant difference in lysozyme activity among treatments (P=0.97), but day 60 lysozyme content was found to be statistically significant (P<0.01), with the BSBM group demonstrating greater activity than the CSPC treatment (P=0.01; Figure 2). Condition (K) values were found to be significant at day 60 (P=0.02), with the FMC diet

providing the highest overall body condition (Figure 3). No differences in K-value were noted at day 0 (P=0.83) and day 15 (P=0.52).

Gut microflora composition

Results from Experiment B showed that Chao-1 (P=0.009) and Shannon (P=0.020) alpha diversity indices were statistically significant among dietary treatments, and demonstrated diet-induced changes in GI microbial communities (Table 6). The FMC group demonstrated a greater Chao-1 value than the BSBM-H (P=0.004), indicating a higher species richness. Conversely, the Shannon H index values for community evenness were lowest in the SBM group, and were significantly less than the BSBM-L (P=0.003) and CSPC (P=0.003) treatments. Similarly, the number of observed species was significant among dietary treatments (P=0.009), and the FMC diet had a greater number of bacterial species than the BSBM-L (P=0.005).

ANOSIM results indicated significant differences among several of the treatments (P<0.001). Differences among these dietary groups are summarized in Table 7. There was no significant differences between the BSBM-L and BSBM-H treatment groups (P=0.404), but all other groups were found to have a significantly different microbiota diversity.

Findings from the SIMPER analysis demonstrated some dissimilarities with the largest dissimilarity observed between the FMC and BSBM-H treatments (70.22% dissimilar; Table 8). From multiple dissimilarity comparisons, it was found that an Acholeplasmataceae (unclassified sugarcane phytoplasma) species was present in the FMC group. This species greatly contributed to the overall dissimilarity between

treatments, accounting for 17.3% of total dissimilarity when compared to the CSPC microbe profile. Species from the Phyla Cyanobacteria, Order Chroococcales, also contributed to the total dissimilarity, with a 22.4% contribution to the compositional differences between the CSPC and BSBM-H groups. Between the SBM and BSBM treatments, an *Exiguobacterium* species contributed 6.8% to total dissimilarity in the BSBM-L group and 7.2% in the BSBM-H treatment. *Peptostreptococcus russellii* was also found to contribute to the dissimilarity composition when comparing the CSPC (6.3 % of total composition) with the BSMB-L (5.3% dissimilar) and BSMB-H (5.7% dissimilar) groups. Between the high and low BSBM inclusion, *Falsiporphyromonas endometrii* contributed 7.1% to the overall dissimilarity. The relative abundances by bacterial Class and Phyla are shown in Figure 4 and Figure 5, respectively.

Discussion

Experiment A: Histological and immunological evaluation

These studies evaluated GIT characteristics and immune responses resulting from processed soybean meals. The bioprocessing of soybean meal has been shown to greatly reduce ANFs and other antigenic factors such as trypsin inhibitors, isoflavones, or conglycinins that may cause GIT complications or degradation (Anderson, 1995; Hei et al., 2012).

Results from the histological evaluation indicated no significant differences in intestinal profile or apparent enteritis resulting from the processed soy diets. In a previous study by Barnes et al. (2014), no significant differences in rainbow trout intestinal morphology were found between 50% and 35% inclusions of a fermented soybean meal

following 205 days of feeding. The current histological analyses appear to corroborate those findings and indicate a lack of physiological malady within the distal trout intestine. Furthermore, due to a production error involving lipid levels during diet manufacture, these results also indicate a lack of negative histological responses from varied dietary lipid levels across all treatments. This reduction in intestinal inflammation may potentially be related to microbial byproducts or structures leftover from the bioprocessing. For instance, a 1% inclusion of galatcooligosaccharides have been found to decrease intestinal alterations caused by plant-based diets in gilthead sea bream (*Diplodus sargus*) at short-term durations (Guerreiro et al., 2016). Furthermore, small inclusions of amino acids, such as arginine and glutamine, have been found to positively impact the intestinal morphology of hybrid striped bass (*Morone chrysops×Morone saxatilis*; Cheng et al., 2012). Small inclusions of beneficial polysaccharides or amino acids may be produced through the manipulation of process conditions or microorganism used in bioprocessing.

Changes to the intestinal leukocyte phagocytosis index was not observed in the short-term samples (day 15). The rainbow trout intestinal segments contain various leukocytes in the mucosal associated lymphoid tissue, including neutrophils and macrophages, which are integral to pathogen detection and removal (Balcazar et al., 2006). Although a quite different gut morphology, it has been found that the posterior intestine in Pacific halibut (*Hippoglossus hippoglossus*) contains the majority of the immune cells, namely IgM+ lymphocytes or macrophages, and are located within the epithelium as opposed to the lamina propria (Grove et al., 2006). The distal intestine serves as an important reservoir for antigen uptake in fish, and the ability to adequately

phagocytize and present antigen within the mucosal tissue is critical in providing protection for the fish (Clerton et al., 1998). These study findings suggest that the bioprocessed or CSPC diets did not impact macrophage phagocytosis, and did not reduce immunocompetency of leukocytes in the rainbow trout hindgut.

Lysozyme activity showed some modulation throughout the feeding trial period, with the BSBM diet producing higher levels in the long-term sample (60 day). A previous study on Atlantic salmon (*Salmo salar*) showed increased lysozyme activity in the intestinal mucosa due to the dietary administration of soybean molasses, indicating a potential inflammatory response and was potential activation of leukocytes (Krogdahl, 2000). Alternatively, as no histological impairment was found in the BSBM treatment groups, these trial results appear to align with findings by Kim and Austin (2006), where high lysozyme activity was found in intestinal mucus samples – stemming from administered probiotics – and indicating an enhancement to gut health. Other studies with probiotic agents have also shown increases in serum lysozyme content, which is likely independent of intestinal inflammation and regarded as an immunopotentiating effect (Saurabh & Sahoo, 2008; Panigrahi et al., 2005). Thus, the administration of BSBM may offer enhanced immune response and potential pathogen protection in the hindgut of rainbow trout.

Immunoglobulin levels were not found to change significantly over the course of the trial. Previous feeding trial studies have evaluated antibody levels in response to feeding soybean-based diets, and antibody increase is generally associated with a GIT inflammatory response (Krogdahl, 2000; Bakke-McKellep, 2000). To date, there has not been a great deal of published results examining the effects of processing or

bioprocessing on immunoglobulin levels in the salmonid GIT. Thus, in future studies, it would be beneficial to further investigate IgM levels to compare to previously reported results, or investigate IgT levels in the distal intestine, an immunoglobulin that has become of recent interest for mucosal immunity in fishes (Zhang et al., 2010).

Experiment B: Gut microflora

The microbial diversity appeared to be dynamic across the dietary treatment groups, with some similarities to previously reported microflora compositions in cultured species. In a previous study evaluating the Atlantic salmon microflora, there were no major differences to community structure reported between BSBM and FM treatment groups, while a SBM group appeared to demonstrate an increase in transient bacteria and decrease in adherent species (Refstie et al., 2006). This was not the case in the current study, as there were significant differences in diversity indices and predominant species resulting from the FMC and BSBM diets, induced by the bioprocessing methodology, ingredient composition or differences in host-specificity among salmonids. For instance, the incorporation of distiller's dried grains with solubles (DDGs) in aquaculture diets has been found to have host-specific implications on the gut microflora while inclusions of wheat gluten in barramundi (*Lates calcifer*) diets demonstrated no negative impact on microflora diversity (Apper et al., 2016; He et al., 2013). In terms of microflora composition, an increase in Firmicutes has been reported in rainbow trout fed plant-based diets and an establishment of the microflora diversity is present following first feeding (Ingerslev et al., 2014a; Ingerslev et al., 2014b). In the current study, there were no significant differences in the Firmicutes composition among treatment groups. There

were overall increased numbers of bacteria belonging to *Bacillus* genera and gammaproteobacteria within the soybean-based diets. Although not determined to be diet-related, *Acetinobacter* (Phyla gammaproteobacteria) populations were also found to be predominant in the microflora of farmed Atlantic salmon (Hovda et al., 2007). Although the gammaproteobacteria phylum contain pathogenic bacteria, it has been found that some of the genera assist digestive function and the ability of the fish to break down chitin (Lan & Love, 2012). It has been previously found that the gammaproteobacteria have been more abundant in rainbow trout fed fishmeal diets, as compared to soybean-based diets, while these study results indicate more gammaproteobacteria in the BSBM treatments (Mansfield et al., 2010). Additional microbial-based components from bioprocessing (i.e. peptides or cell wall components) requires further investigation to determine the influence of these factors on GIT microflora diversity, as it has been reported that the direct inclusion of some probiotic strains do not always impact fish GIT colonization (Lamari et al., 2013). Further, it has been found that microbiota communities and populations within the distal and proximal intestine differ because of gastric conditions, and proximity to digestive enzyme activities, both of which may be modulated by diet type (Lyons et al., 2015).

The probiotic or pathogenic mechanisms of the microbiota are still being investigated and pertinent species of bacteria have been identified as beneficial or non-beneficial. For example, *Photobacteria*, *Aeromonas*, *Yersinia*, *Pseudomonas*, *Vibrio*, and *Flavobacterium* are all recognized to be pathogenic to fishes, but low levels in the healthy gut have been found to be non-pathogenic (Dehler et al., 2016). Other beneficial bacteria species, such as *Weisella* (0.27% total composition of the BSBM-L group) and

Carnobacteria (0.67% total composition of the CSPC group) with probiotic effects (Dehler et al. 2016). Lactic acid bacteria species (i.e. may exert their beneficial effects via inhibition of metabolites, competition, or immunostimulation via intrinsic signaling or metabolic byproducts (Vázquez et al., 2005). Further, *Cetobacterium* species have been reported to exert a beneficial function by assisting with gut fermentation, enzyme metabolism, and vitamin production (Lyons et al., 2015).

The microbiota has also been found to influence the immunological parameters and fish health. For example, rainbow trout diets supplemented with *Bacillus* have been found to increase the predominance of circulating leukocytes, and potentially advocating the importance of the reestablishment of the GIT microflora following antibiotic treatments (Merrifield et al., 2010). Further, it many enzyme-secreting species are currently being characterized for their contributions to immunostimulation or pathogen defenses, and nutrient uptake (Ray et al., 2012). As many of these microflora-related mechanisms are still being explored, the gut microflora components can enhance innate immunity, elicit antimicrobial peptides (AMPs), and compete against fish pathogens (Gomez & Balcazar, 2008). Thus, further evaluation of bioprocessed plant feedstuffs in pathogen challenge or short-term feeding trials may elucidate the microflora dynamics with respect to innate and mucosal immune parameters.

Although there has been much progress in comprehending and classifying the fish microflora with respect to diet types, there is much to be studied to understand the importance of gut health in more detail. Further, research is also being conducted to better understand the epithelial microbiome of fish skin to better understand these complex microbial community interactions and mechanisms (Lowrey et al., 2015). There

have been many advancements made for salmonid culture in the past decades, and this has been evidenced with the BSBM and CSPC inclusions maintaining healthy gut histology throughout the trial duration, in this particular study. As the aquaculture industry expands and looks for novel protein sources as fishmeal replacements, the importance of the GIT microbiota may become of importance for the maintenance of healthy fish stocks.

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Tables

Table 1. Ingredient proximate composition of experimental protein sources (as is basis) used in both feeding trials. Fish meal control = FMC; soybean meal = SBM; commercial soy protein concentrate = CSPC; bioprocessed soybean meal = BSBM.

Proximate	FMC	SBM	CSPC*	BSBM
Crude Protein (g $100g^{-1}$)	65.54	45.50	65.00*	63.80
Crude Lipid (g $100g^{-1}$)	8.50	1.71	1.00*	0.56
Crude fiber (g $100g^{-1}$)	0.10	5.16	3.50*	7.31
Ash (g $100g^{-1}$)	18.74	6.02	6.5*	3.80
Moisture (%)	8.01	11.90	7.00	4.93

*Specifications from manufacturer, ADM (Chicago, IL)

Table 2. Experiment A diet formulations and analyzed proximate composition of trial diets. Fish meal control = FMC; soybean meal = SBM; commercial soy protein concentrate = CSPC; bioprocessed soybean meal = BSBM.

Ingredient (g 100 g^{-1} dmb)	FMC	CSPC	BSBM
Menhaden fishmeal[a]	38.10	9.97	9.97
CSPC[b]	0.00	27.91	0.00
BSBM [c]	0.00	0.00	27.91
Whole cleaned wheat[d]	18.04	17.94	17.94
Empyreal[e]	8.02	7.97	7.97
Poultry meal[f]	5.01	4.98	4.98
Feathermeal[f]	5.01	4.98	4.98
Dicalcium phosphate[g]	1.91	1.89	1.89
Blood meal[h]	4.42	4.98	4.98
Vitamin premix[i]	1.00	1.00	1.00
Mineral premix[j]	1.00	1.00	1.00
Stay-C[k]	0.10	0.10	0.10
Lysine[l]	1.20	1.20	1.20
Methionine[l]	0.40	0.40	0.40
Choline[m]	0.20	0.20	0.20
Taurine[n]	0.50	0.50	0.50
Astaxanthin[o]	0.04	0.04	0.04
Soybean oil[p]	8.02	7.97	7.97
Fish oil[q]	7.02	6.98	6.98
Totals	100.00	100.00	100.00
<u>Proximate Composition</u>			
Crude Protein	55.60	50.28	51.46
Crude Lipid	9.59	5.99	4.99
Crude Fiber	1.06	2.22	3.33
Ash	14.01	9.70	7.42
Moisture	4.04	4.78	3.80

[a] Special Select, Omega Protein, Houston, TX; [b] Soycomil P, ADM, Chicago, IL; [c] Prairie AquaTech, Brookings, SD; [d] AgFirst Farmers Cooperative, Brookings ,SD; [e] Cargill Corn Milling, Blair, Nebraska; [f] Tyson Foods, Springdale, AR; [g] Feed Products Inc., St. Louis, MO; [h] Mason City By-Products, Mason City, IA; [i] ARS 702 premix, Nelson and Sons, Murray, UT; [j] ARS 640 trace mix, Nelson and Sons, Murray, UT; [jk]DSM Nutritional Products, Parsippany, NJ; [kl]Pure Bulk, Roseburg, OR; [m] BalChem Corporation, New Hampton, NY; [n] Nutra blend LLC, Neosho, MO; [o] ChemSol, Minnetonka, MN; [p] South Dakota Soybean Processors, Volga, SD; [q] Daybrook Fisheries, Empire, LA.

Table 3. Experiment B diet formulations and analyzed proximate composition of trial diets. Fish meal control = FMC; Soybean meal = SBM; commercial soy protein concentrate = CSPC; bioprocessed soybean meal = BSBM.

Ingredient (g 100 g^{-1} dmb)	FM	SBM	CSPC	BSBM-L	BSBM-H
Menhaden fishmeal[a]	15.00	8.00	12.00	12.00	8.00
Soycomil-P[b]	0.00	0.00	25.00	0.00	0.00
BSBM[c]	0.00	0.00	0.00	25.00	35.54
SBM[d]	0.00	35.00	0.00	0.00	0.00
Wheat midds[e]	18.00	7.96	13.25	13.25	11.26
Whole cleaned wheat[f]	18.00	10.00	17.94	15.60	14.87
Empyreal[g]	5.00	5.00	0.00	0.00	0.00
Poultry meal[h]	14.32	8.00	5.00	5.00	4.00
Feathermeal[h]	7.50	4.00	7.50	7.50	5.00
Blood meal[i]	5.00	3.00	4.98	2.50	2.00
Vitamin premix[j]	1.25	1.25	1.25	1.25	1.25
Mineral premix[k]	1.50	1.35	1.50	1.50	1.75
Stay-C[l]	0.02	0.02	0.02	0.02	0.02
Lysine[m]	2.10	1.80	2.00	2.00	2.00
Methionine[m]	0.75	0.60	0.75	0.75	0.75
Choline[n]	0.06	1.00	0.60	0.60	0.06
Fish oil[o]	11.50	13.02	12.85	12.85	13.50
Totals	100.00	100.00	100.00	100.00	100.00
Proximate Composition (g 100 g^{-1} dmb)					
Crude Protein	44.47	41.14	41.26	41.53	41.87
Crude Lipid	19.50	14.36	14.55	11.11	10.80
Crude Fiber	2.67	3.09	3.61	3.81	3.79
Ash	8.02	7.70	8.22	7.96	7.11
Moisture	5.50	4.60	4.45	4.60	5.53

[a] Special Select, Omega Protein, Houston, TX; [b] Soycomil DM ; [c] Prairie AquaTech, Brookings, SD; [d] South Dakota Soybean Processors, Volga, SD; [e] Consumers Supply Distributing, Sioux City, IA; [f] AgFirst Farmers Cooperative, Brookings, SD; [g] Cargill Corn Milling, Blair, Nebraska; [h] Tyson Foods, Springdale, AR; [i] Mason City By-Products, Mason City, IA; [j] ARS 702 premix, Nelson and Sons, Murray, UT; [k] ARS 640 trace mix, Nelson and Sons, Murray, UT; [l] DSM Nutritional Products, Parsippany, NJ; [m] Pure Bulk, Roseburg, OR; [n] BalChem Corporation, New Hampton, NY; [o] Virginia Prime Gold, Omega Protein, Houston, TX.

Table 4. Histological scoring system for rainbow trout intestines in experiment A (modified and adapted from Barnes et al. 2014.)

Score	Appearance
	Lamina propria of simple folds
1	Thin and delicate core of connective tissue in all simple folds
2	Lamina propria slightly more distinct and robust in some folds
3	Clear increase in lamina propria in most simple folds
4	Thick lamia propria in many folds
5	Very thick lamia propria in many folds
	Connective tissue between base of folds and stratum compactum
1	Very thin layer of connective tissue between base of folds and stratum compactum
2	Slightly increased amount of connective tissue beneath some mucosal folds
3	Clear increase of connective tissue beneath most mucosal folds
4	Thick layer of connective tissue beneath many folds
5	Extremely thick layer of connective tissue beneath some folds
	Vacuolization
1	Large vacuoles abundant and present in most epithelial cells
2	Large vacuoles numerous
3	Increased number of large vacuoles
4	Very few large vacuoles present
5	Large vacuoles absent

Table 5. Gut leukocyte phagocytosis rate at day 15 and total immunoglobulin (Ig) content of distal intestine homogenate at days 15 and 60, in experiment A. Fish meal control = FMC; Soybean meal = SBM; commercial soy protein concentrate = CSPC; bioprocessed soybean meal = BSBM.

Immune Parameter	FMC	CSPC	BSBM
Leukocyte phagocytosis (%)	56.20±6.65	49.65±6.40	43.89±6.07
Total Ig at day 15 (mg mL^{-1})	2.57±0.48	1.94±0.33	1.74±0.48
Total Ig at day 60 (mg mL^{-1})	2.90±0.43	1.79±0.41	2.46±0.36

Table 6. Alpha diversity indices at for dietary treatment groups at day 60. Values are derived means from 5 replicates per dietary treatment, depicted with standard error of the mean (SEM). Different letters denotes statistical significance between treatments.

	Chao-1	**Shannon**	**Observed Species**
FMC	150.4±18.0[a]	3.1±0.1[ab]	146±17[a]
SBM	102.7±13.0[ab]	2.8±0.1[b]	100±13[ab]
CSPC	116.0±8.5[ab]	3.4±0.0[a]	111±8[ab]
BSBM-L	122.8±9.0[ab]	3.4±0.1[a]	121±8[ab]
BSBM-H	77.7±11.6[b]	3.2±0.1[ab]	76±11[b]

Table 7. ANOSIM pairwise tests for experiment B, with Bonferroni adjustments and using the Bray-Curtis similarity index, for microbiota diversity similarities between dietary treatments at day 60 ($P<0.001$, $R=0.3069$). Fish meal control=FMC, Soybean meal=SBM, Commercial soy protein concentrate=CSPC, Bioprocessed soybean meal=BSBM.

	FMC	**SBM**	**CSPC**	**BSBM-L**	**BSBM-H**
FMC	-				
SBM	0.008	-			
CSPC	0.009	0.008	-		
BSBM-L	0.008	0.007	0.014	-	
BSBM-H	0.007	0.008	0.009	0.4042	-

Table 8. Average microbiota dissimilarities between experiment B dietary treatment groups at day 60, as calculated using SIMPER analysis. Fish meal control=FMC, Soybean meal=SBM, Commercial soy protein concentrate=CSPC, Bioprocessed soybean meal=BSBM.

	FMC	**SBM**	**CSPC**	**BSBM-L**	**BSBM-H**
FMC	-				
SBM	59.97	-			
CSPC	54.76	47.65	-		
BSBM-L	62.10	62.72	51.98	-	
BSBM-H	70.22	65.03	58.11	62.95	-

Figures

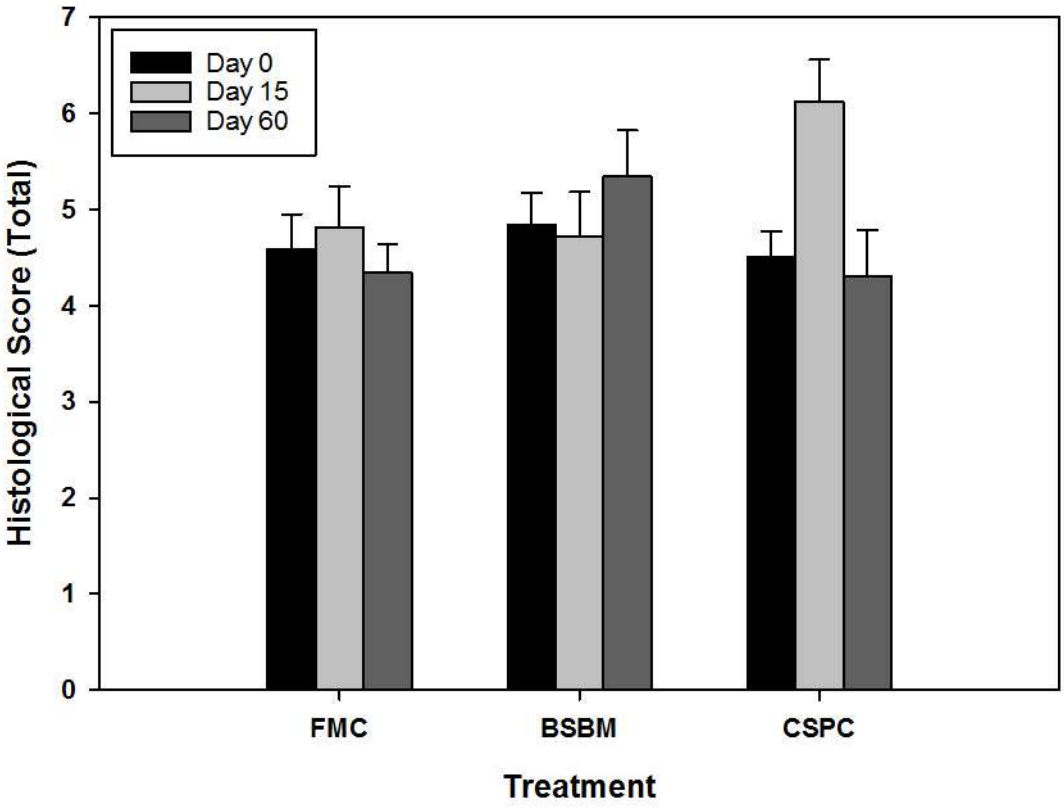

Figure 1. Histological assessment of rainbow trout distal intestines from experiment A on day 0 (P=0.75), 15 (P=0.08), and 60 (P=0.22) based on a modified scoring methodology (Barnes et al. 2014). Fish meal control = FMC; Soybean meal = SBM; commercial soy protein concentrate = CSPC; bioprocessed soybean meal = BSBM.

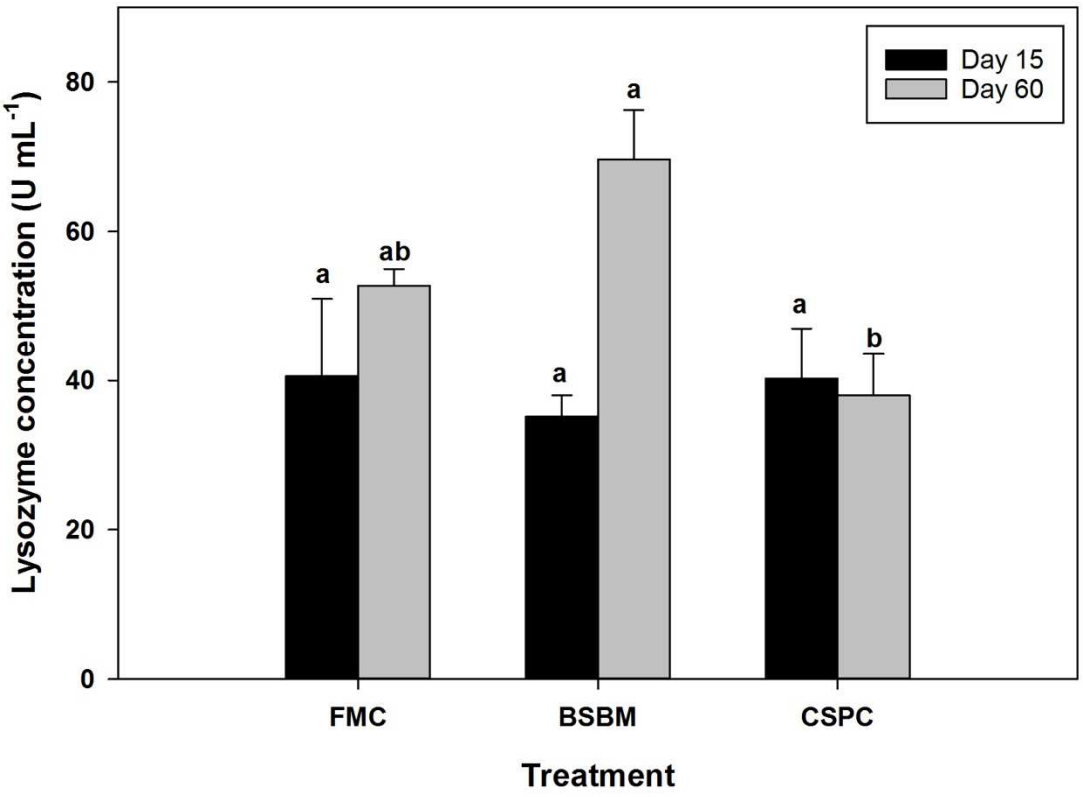

Figure 2. Homogenate lysozyme levels of rainbow trout intestines sampled at day 15 and day 60 in experiment A. Different letters (a, b, c) signify significant differences ($P < 0.05$). Fish meal control = FMC; Soybean meal = SBM; commercial soy protein concentrate = CSPC; bioprocessed soybean meal = BSBM.

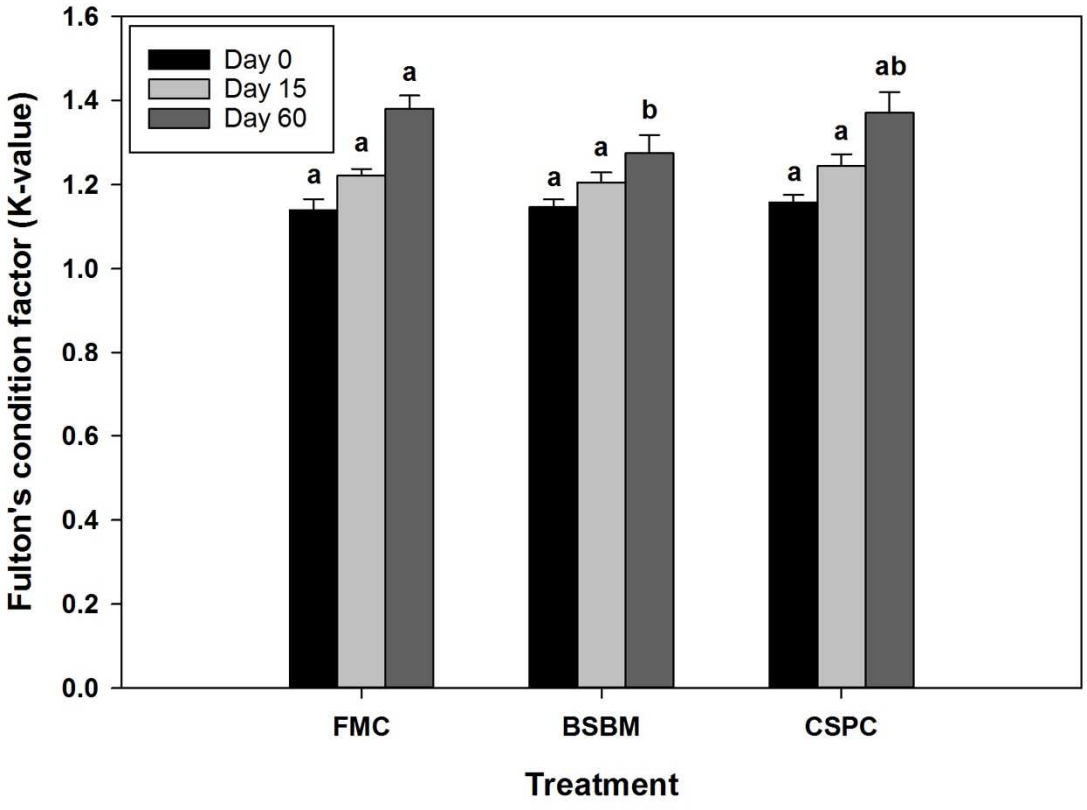

Figure 3. Fulton's condition factor (K-value) at day 0, 15 and 60 from experiment A. Different letters denote a statistical significance among treatments. Different letters (a, b, c) signify significant differences ($P < 0.05$). Fish meal control = FMC; Soybean meal = SBM; commercial soy protein concentrate = CSPC; bioprocessed soybean meal = BSBM.

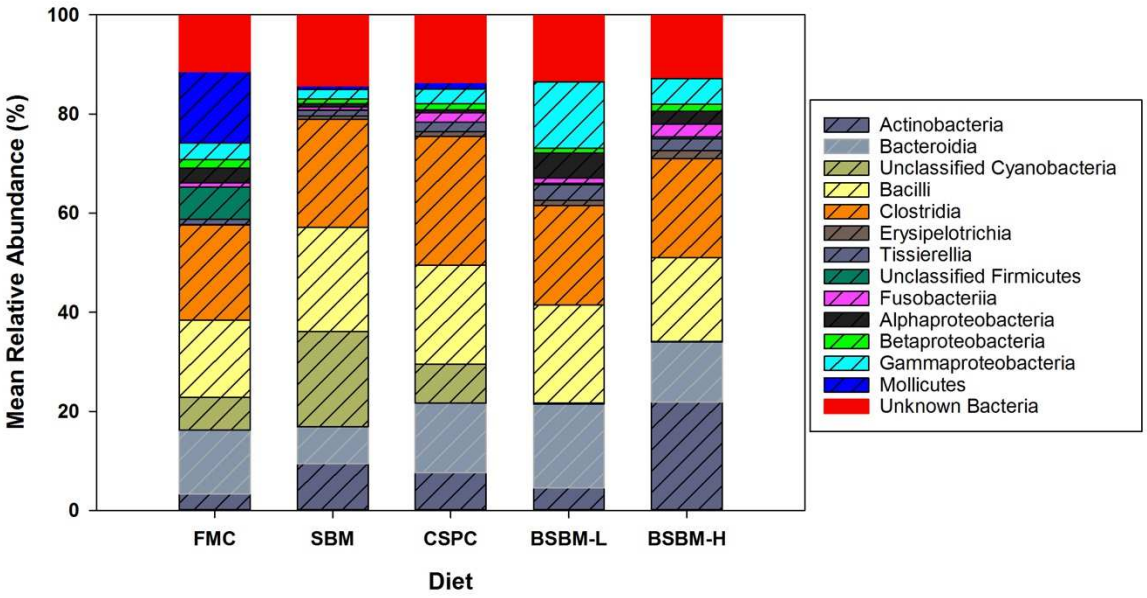

Figure 4. Relative abundance of microbiota, by bacterial taxa, obtained in experiment B from rainbow trout fecal material at day 60. Fish meal control = FMC; Soybean meal = SBM; commercial soy protein concentrate = CSPC; bioprocessed soybean meal = BSBM.

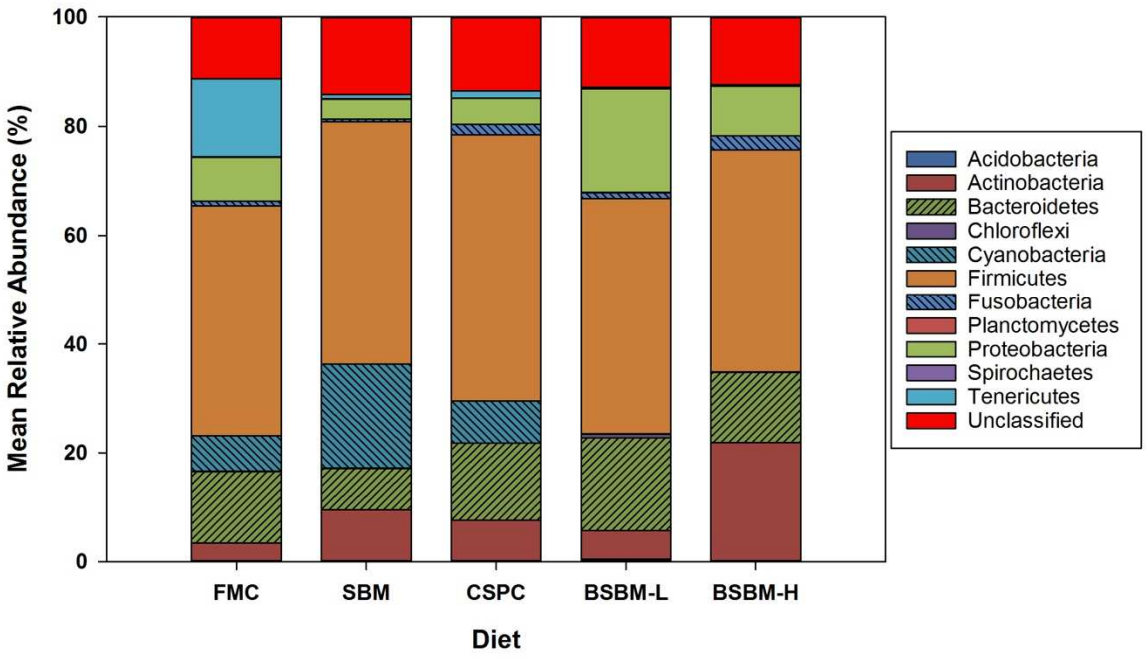

Figure 5. Relative abundance of microbiota, by bacterial phyla, obtained in experiment B from rainbow trout fecal material at day 60. Fish meal control = FMC; Soybean meal = SBM; commercial soy protein concentrate = CSPC; bioprocessed soybean meal = BSBM.

CHAPTER 6. ACUTE STRESS RESPONSE IN RAINBOW TROUT *Oncorhynchus mykiss* FED BIOPROCESSED SOYBEAN DIETS

Abstract

The use of plant-based proteins to replace marine-derived fish meal (MDFM) has gained momentum as the nutritional quality of processed plant ingredients improves and the aquaculture industry seeks lower feed costs while sustaining production efficiency. As a result of bioprocessing, additional fish health benefits may also result from these ingredients and may influence the fish stress response, a common concern for many culturists. A 60-day feeding trial was conducted to evaluate physiological changes from acute stressors in rainbow trout (*Oncorhynchus mykiss*) fed soy-based diets. Experimental diets contained two bioprocessed soybean meal levels (BSBM; 25% [low] and 35% [high] inclusions) or a commercial soy protein concentrate (CSPC; 25% inclusion). Dietary controls included a fish meal (FM) reference and a defatted soybean meal (SBM; 35% inclusion). Juvenile rainbow trout (42.3g) were stocked at 15 fish/tank in a 30-tank recirculating aquaculture system and fed at 2% of body weight for the 60-day trial. Naïve rainbow trout were sampled on day 0 for baseline levels of cortisol, blood glucose, hematocrit, and macrophage respiratory burst activity. On days 30 and 60, 5 fish per tank were subjected to 4 hours of acute stressors (low flow rate, decreased dissolved oxygen and handling stressors) or left unstressed as controls for respective diet treatments. Day 30 changes in macrophage RBA were significantly different among diet treatments in the stressed ($P=0.004$) groups and between the stressed and unstressed groups at Day 60 ($P=0.004$). Within the stressed treatments, the BSBM low inclusion

diets were demonstrated significantly less RBA than the BSBM high inclusion (P=0.002), CSPC (P=0.049), and SBM treatments (P=0.017). For plasma cortisol, there were no dietary treatment differences but day 30 stressed (11.48±1.51 ng mL^{-1}) and unstressed groups (0.47±0.28 ng mL^{-1}) had a significant difference in group means (P<0.001). Day 60 plasma cortisol levels following a similar trend, significance between stressed (7.22±1.48 ng mL^{-1}) and unstressed (0.36±0.12 ng mL^{-1}) groups (P<0.001). Continued assessment of novel plant-based ingredients to reduce stressors may benefit producers by allowing increased stocking densities and reducing losses due to fish transport, thus further contributing to the needs of a rapidly expanding domestic aquaculture market.

Introduction

Aquaculture is rapidly expanding in the United States, with an estimated production of 449, 441 (U.S) tons, and value of $1.14 billion in 2014 (FAO 2016). Innovations in the processing of seed meals, particularly soybean meal (SBM), are contributing to industry growth by reducing dependency on marine-derived fish meal (MDFM), the primary protein ingredient in aquafeeds (Rossi et al., 2016; Barnes et al., 2015; Refstie et al., 2006). Microbial bioprocessing of SBM provides increased protein content and reduced anti-nutritional problems, more comparable to MDFM. Additionally, the bioprocessed SBM (BSBM) provides the potential to modulate innate immune parameters (Barnes, 2014; Trushenski et al., 2014; Kokou, 2012). Thus, these microbially enhanced ingredients may contribute to fish health.

As producers increase fish stocking densities and require transportation or relocation throughout production stages, stressors are imposed which impact fish health.

The fish stress response to acute stressors, that is high intensity stressors administered over a short time period, often involves primary and secondary responses that actuate and enable the ability of the fish to cope with short-term stress (Tort, 2011; Barton, 2002). The primary response involves catecholamine release and neuroendocrine modulation, while the secondary response involves circulating mineral and glucose fluctuations (Lays et al., 2009). The immune system function and reproductive ability are often compromised if the stressor becomes chronic or has inducted the fish stress response into the tertiary phase (Barton, 1997). Stress responses can be induced by simple mechanisms such as handling, poor water quality, and stocking densities in commercial production systems (Conte, 2004). It has been found that there is a genetic predisposition to stress response, namely higher heritability for cortisol than glucose responses, and new branches of stress research have become of interest with the establishment of selective breeding programs (Pottinger & Carrick, 1999; Fevolden et al., 1993). As the aquaculture sector expands and increases production capacities, the welfare of cultured fishes is important to maintaining physiological functions and optimal growth potential (Ashley, 2007).

Reducing stress levels can mitigate issues such as decreased feed consumption, conversion, and growth and improve survival rates following a stress event (Ardiansyah & Fotedar, 2016; Schreck et al., 2001; Pankhurst, 1997). Feed composition can have an effect on the fish stress response, with variations in amounts and quality of proteins, lipids, and micronutrients accounting for changes in physiological parameters (Fletcher, 1997). The use of plant proteins may elicit intrinsic effects and can reduce stress-related gene expression, as found in Senegalese sole (*Solea senegalensis*; Batista et al., 2016).

Further, the incorporation of soybean meal (SBM) in sunshine bass diets (*Morone chrysops* × *M. saxatilis*), resulted in diet-induced changes to blood glucose levels following manipulated tank stressors (Laporte & Trushenski, 2012). Microbial constituents, such as β-glucan and mannan oligosaccharides found in yeast cells, were found to cause changes to plasma glucose and cortisol levels in channel catfish (*Ictalurus punctatas*) subjected to a low water volume stressors (Welker et al., 2007). These findings demonstrate the potential for bioprocessed byproducts to enhance physiological stress parameters in cultured species. The ability to control and understand dietary composition, especially from the primary protein ingredients, is an important factor in reducing stress within production systems. Thus, this study aimed to characterize the rainbow trout stress response to acute stressors, following the administration of soy-based diets.

Materials and Methods

Experimental Design and Feeding

A 60-day feeding trial with juvenile rainbow trout was designed to investigate the impact of acute stressors on endocrinological, physiological and immunological metrics. The feeding trial incorporated five isonitrogenous diets that were formulated to meet the nutritional requirements of rainbow trout (NRC 2011). Two of the experimental diets contained either high or low inclusions of bioprocessed soybean meal (BSBM) as a main protein source, which replaced 46% and 20% of the dietary FM, respectively. A commercial soy protein concentrate (CSPC) was also included at a 25% inclusion level. Experimental diets compared against fish meal (FM) and soybean meal (SBM) reference

diets. Each treatment was allocated to six replicate tanks, with three replicates designated for stress treatments. Analyses of primary protein sources were completed by contracted certified private laboratories for crude protein (AOAC 2006, method 990.03), crude fat (AOAC 2006, method 990.03), crude fiber (AOAC 2006, method 978.10), moisture (AOAC 2006, method 934.01), and ash (AOAC, method 942.05) and amino acids (AOAC 2006, method 982.30 E(a,b,c)). Proximate compositions of the experimental protein sources are summarized in Table 1, while the dietary formulations and composition analyses are presented in Table 2.

Diets were prepared by grinding the large particle ingredients with a Fitzpatrick Comminutator (Elmhurst, IL) with 1.27 mm screen prior to dry blending. Dry blended feedstuffs were then transferred to a ribbon mixer (Patterson Equipment, Toronto, ON) and diets were extruded using an Extru-Tech E325 single-screw extruder (Sabetha, KS). Extruded pellets were dried with a conveyor oven drier (Colorado Mill Equipment, Canon City, CO), screen-sifted with a Rotex screener (Rotex Inc., Cincinnati, OH) and then lipid coated using a Phlauer vacuum coater (A&J Mixing, Oakville, Ontario). Subsequently, the diets were cooled to room temperature, bagged, and stored at 4°C, pending use.

Four hundred and fifty juvenile rainbow trout (42.30±0.26 g) were randomly stocked into 30 circular, 114L tanks connected in parallel to a recirculating aquaculture system (RAS). The randomly assigned study tanks received dechlorinated make-up water and the RAS was kept at suitable conditions for rainbow trout culture (15.3°C, 7.0 mg L^{-1} dissolved oxygen, pH 8.3). Fish were fed 2% of body weight per day (8 am and 4pm), twice daily, and feed rates were adjusted following tank biomass measurement at

day 30. Feeding rates were calculated based on initial tank biomass at day 1, and incorporated a projected SGR of 2.50% and projected FCR of 1.2. Feeding rates were adjusted daily to account for any mortalities. No palatability or feeding issues were noted across any of the dietary treatments. Fish were found to be healthy during the entire trial, with the exception of 10 mortalities noted over the 60 days, due to jumping out of tanks, unknown illness, or reduced tank flow in one instance. Thirty fish were sampled at day 0 to obtain baseline metrics for the trial prior to being exposed to acute stressors at day 30 and day 60, leaving 14 fish per tank for the trial.

On the two stress sampling dates, 5 fish per tank were euthanized using 250 ppm of tricaine methanesulfonate (MS 222). Fish were sampled for weight, length, spleen, and head kidneys to calculate physiological metrics and spleen-somatic index (SSI). Blood was collected using a caudal vein sever, pooled for five fish per tank, and then deposited into a heparinized BD Vacutainer (Franklin Lakes, NJ). The blood samples were briefly held on ice and then centrifuged at 1000 x g for 10 minutes to isolate plasma. Isolated plasma was then frozen at -80°C until required for the cortisol and ion assays. At the time of blood collection, a drop of fresh whole blood was analyzed using a ReliOn Prime glucose meter (Bentonville, AR). For hematocrit sampling, whole blood was placed in a heparinized hematocrit tube (Fisher Scientific, Waltham, MA) and spun at 12,000 RPM for 10 min in a microcentrifuge (ThermoFisher Sorvall Legend 17, Waltham, MA) equipped with hematocrit rotor.

To determine relative growth (RG) and specific growth rate (SGR) at the end of the trial, average fish weight was calculated from the 5 fish per tank sampled at the trial endpoint and compared to the average weight of the fish determined for the starting tank

biomass. The tank biomass gain was calculated as the difference in final tank biomass from the starting tank biomass, taking into account any mortalities and sampled fish weights from the day 30 and day 60 necropsy. Growth performance, condition factor (K), and SSI at day 60 were calculated as:

$$\text{SGR} = \frac{\ln(\text{final wt (g wet)}) - \ln(\text{start wt (g wet)})}{\text{n (days)}} \times 100$$

$$\text{RG} = \frac{\text{final wt (g wet)} - \text{start wt (g wet)}}{\text{start wt(g wet)}} \times 100$$

$$\text{Tank biomass gain (g)} = \text{final biomass (g)} - \text{starting biomass (g)}$$

$$\text{K} = \frac{\text{final weight (g wet)}}{\text{total length (mm)}^3} \times 100{,}000$$

$$\text{SSI} = \frac{\text{fresh spleen weight (g)}}{\text{whole fish weight (g wet)}} \times 100$$

Acute Stressors

On each stress sampling date, 5 fish from each of the 3 stressed group tanks were relocated to a separate system to receive the acute stressors. Within the stress treatment system, tank volumes were reduced to approximately 1/3 volume (approximately 37 L), flow was stopped, and low dissolved oxygen (DO) conditions were created by reducing aeration. Water temperature and dissolved oxygen parameters were monitored at a minimum of twice per hour to quantify stressor parameters (Day 30=17.34°C, 3.88 mg L^{-1}; Day 60=15.05°C, 4.38 mg $L^{-}1$). Further, fish were handled with a net for 1min intervals, every half hour, for 4 hours. The beginning of the stress treatments was staggered at 5 minute intervals between tanks to establish equal sampling time intervals. The unstressed fish groups for each dietary treatment were sampled directly from the

remaining 3 tanks that were not selected for the stress treatment. Thus, the unstressed served as a control group to compare baseline stress responses, while the FM and SBM treatments served as dietary control groups.

Head kidney macrophage isolation and respiratory burst activity (RBA)

Macrophages were isolated using established methods by Secombes et al (1990), with modifications. Head kidney isolates were removed aseptically and stored on ice in 2mL of Leibovitz-15 (L-15) medium containing 2% fetal calf serum (FCS) (Sigma-Aldrich, St. Louis, MI), 100 i.u. penicillin-streptomycin mL^{-1} (Sigma-Aldrich), and 10 units of heparin mL^{-1} (Fisher Scientific, Waltham, MA). Pronephros samples were processed within 24h of extraction and the tissues were passed through 100μm mesh in 2mL of the modified L-15 medium. The samples were then placed on a 34%/51% Percoll (Fisher Scientific, Waltham, MA) density gradient and centrifuged at 500 x g for 30min. Cells were collected at the density interface and added to cold modified L-15 medium. The collected cells solutions were then centrifuged at 1000 x g for 10 min and the pellet was re-suspended in L-15 supplied with 0.1% FCS. Samples were adjusted to $2x10^7$ cells mL^{-1} using a hemocytometer, verified for viability using trypan blue, and $2x10^6$ cells were seeded in each well of a 96 well tissue culture plate. Cells were allowed to adhere for 2h at 18°C before being washed with fresh L-15 (non-supplemented). Adherent cells were incubated overnight in L-15 supplemented with 5% FCS prior to being used in the assays. Macrophage respiratory burst activity (RBA), derived from the pronephros was determined using spectrophotometric analysis of nitroblue tetrazolium (NBT) reduction via superoxide production. For the RBA reagent preparation, NBT was added at a

concentration of 1mg mL^{-1} and 1µg mL^{-1} phorbyl 12-myristate 13-acetate was added to L-15 medium. 100µL of the RBA reagent was added to the wells and allowed to react for 30min. The wells were then emptied, the cells fixed with methanol, and the wells were washed twice with a 70% methanol rinse. 120µL of 2M potassium hydroxide (KOH) and 140µL of dimethyl sulfoxide (DMSO) were added to each well and the plates were agitated and read in a microplate reader (BioTek Corp., Winooski, VT) at 620nm, using KOH/DMSO wells as blanks. OD_{620} readings were adjusted for blanks and were compared to the respective RBA.

Plasma cortisol and ion analyses

Serum concentrations of cortisol were determined in duplicate using the ImmuChem Coated Tube Cortisol kit (MP Biomedicals, Solon, OH). All samples were done in a single assay with an intra-assay CV of 5.2%. Sensitivity of the assay was 0.16 mg/dL. Plasma ion concentrations were analyzed using a Radiometer ABL 77 (Radiometer, Brea, CA). A 70µL aliquot of trout plasma was loaded into an ABL electrolyte sensor cassette, cassettes were calibrated with the manufacturer's standards solution, and analyzed for Na^{+}, K^{+}, Cl^{-}, and Ca^{+2} concentrations.

Statistical analyses

JMP 12 (SAS Corporation, Cary, NC) and Program R (R Core Development Team, Vienna, Austria) were used to perform data analysis of trial results. Data were Analysis of variance (ANOVA) was used to compare immune metrics, while Tukey's HSD was used for multiple comparisons, when applicable. Nonparametric data were

analyzed with the Kruskal-Wallis one-way analysis by rank and Mann-Whitney U tests, incorporating the Bonferroni correction, for post-hoc comparisons. The independent t-test was used to compare means between stressed and unstressed groups. Analysis of Covariance (ANCOVA) incorporated the metrics from the unstressed group as a covariate. Results from statistical analyses were deemed significant at $\alpha=0.05$.

Results

Growth, Condition Factor, and Spleen Somatic Index

No significant differences were found in RG (P=0.057), SGR= (P=0.057) or biomass gain (P=0.302; Table 3). K values were different between treatment groups at day 30 (P=0.022; Figure 1). Between dietary treatments, the fish fed the SBM and BSBM-H diets were found to have greater K-values than the FM control diet (P=0.038 and P=0.042, respectively), although the CSPC diet had the greatest RG, SGR, and biomass gain. The overall body condition was also found to be significant among dietary treatment groups at the day 60 sampling (P=0.005), where K for the BSBM-H group was again significantly greater than the FM reference (P=0.035). There were no differences found for the SSI at day 30 (P=0.299) and day 60 (P=0.055) sampling time points, although mean SSI was found to be highest in the BSBM-L (0.123) and BSBM-H (0.123) treatments (Table 3).

Plasma Glucose

Day 30 plasma glucose levels were not significant among dietary treatments within unstressed (P=0.506) and stressed groups (P=0.311; Figure 2). Fish subjected to

stressors displayed blood glucose levels significantly higher (94.59±3.43 mg dL^{-1}) than the unstressed fish (73.05±2.81 mg dL^{-1}; P<0.001). Diet-related changes in glucose were observed among the stressed fish at day 60 (P=0.006), with the SBM group having a significantly greater glucose concentration than the BSBM-H group (P=0.016). There were no significant treatment differences among the unstressed fish (P=0.774), nor between stressed (71.43±1.51 mg dL^{-1}) and unstressed groups (69.28±1.15 mg dL^{-1}; P=0.260) at day 60.

Plasma cortisol

No differences in dietary treatment group cortisol levels were observed within either the unstressed (P=0.402) and stressed (P=0.669) groups at day 30 (Table 4). Day 60 findings were similar, with no differences among dietary treatments in the unstressed (P=0.362) and stressed (P=0.320) groups. When the average plasma cortisol concentrations of unstressed (0.47±0.28 ng mL^{-1}) and stressed (11.48±1.51 ng mL^{-1}) groups were compared at day 30, there was a significant difference in group means (P<0.001). Day 60 results followed a similar pattern and cortisol was significant between unstressed (0.36±0.12 ng mL^{-1}) and stressed (7.22±1.48 ng mL^{-1}) groups (P<0.001). Thus, there appeared to be some secondary stress modulations between unstressed and stressed subgroups, but no apparent diet effects on the cortisol concentrations.

Respiratory Burst

Day 30 changes in macrophage RBA were significantly different among diets in the unstressed (P<0.001) and stressed fish (P=0.004; Figure 4). Within the unstressed

diet treatments, no significant difference was found between the SBM and BSBM-L groups (P=0.334). Similar findings were noted among treatments in the stressed group, as the BSBM-L fish demonstrated significantly lower RBA than the BSBM-H (P=0.002), CSPC (P=0.049), and SBM treatments (P=0.017). Although there were differences among dietary treatments, unstressed and stressed groups were not found to be statistically different (P=0.394) at day 30. Conversely, on day 60, unstressed and stressed group means were significantly different (P=0.004), and the stressed group showed an overall decrease in RBA, with the exception of the CSPC treatment. Analysis of treatment effects did not yield any differences among the stressed diet groups, but the unstressed dietary treatments were different (P<0.001) and the CSPC group had a lower RBA and reduced superoxide production than did the BSBM-L (P=0.040), BSBM-H (P=0.040), and SBM (P<0.001) groups.

Hematocrit

Day 30 hematocrit values were found to be significant among treatments in the unstressed group (P=0.029), while the stressed fish had no significant treatment differences (P=0.190; Figure 3). The CSPC treatment had the greatest hematocrit level, significantly greater than the FM group (P=0.049). When unstressed and stressed groups were compared at day 30, the unstressed fish averaged a significantly greater mean hematocrit value (35.67±0.54 %) than the stressed group (33.01±0.87 %; P=0.005). The same pattern was observed between unstressed and stressed groups at Day 60, with the unstressed fish again having a significantly greater blood hematocrit (39.4±0.33 %) than

the stressed fish (34.82±0.43 %; P<0.001). There were no observed differences across dietary treatments among the unstressed (P=0.262) and stressed (P=0.543) groups.

Plasma ion concentrations

Na^+ concentrations were shown to decrease in the stressed group at day 30 (P<0.001) and day 60 (P=0.013; Figure 5). K^+ values were also highest in fish from within the stressed group at day 30 (P=0.003), while plasma Cl^- concentrations were significantly reduced in the stressed group at both day 30 (P<0.001) and day 60 (P=0.006). Although Ca^{+2} concentrations were higher in the unstressed fish, they were not significant at day 30 (P=0.625) or day 60 (P=0.653). In terms of dietary treatment differences, the only difference observed was in Ca^{+2} concentrations in the stressed day 60 fish (P=0.014), with SBM Ca^{+2} concentrations elevated in comparison to the other dietary treatments. Ca^{+2} in the unstressed groups was a significant covariate (P=0.016), but the treatment effect was not significant (P=0.123).

Discussion

Overall, trial results showed diet-related responses accrued during the 60-day period, including stress-induced changes to physiological and immunological parameters. Findings in cortisol measurements showed an overall increase in cortisol in the stressed group, but there was no apparent indication of diet-related differences. At the day 30 stress event, all of the bioprocessed soy products elicited a relatively high cortisol response, but the CSPC product was the only treatment group resulting in elevated cortisol by day 60, although not found to be statistically significant. In a previous study

with European seabass (*Dicentrarchus labrax*), probiotic administration was found to decrease cortisol levels in whole body homogenates (Carnevali et al., 2006). Although no probiotics were included in this trial, the microbial-based BSBM diets may account for the decreased cortisol levels in comparison to the extraction processes used to produce CSPC. Further, the amino acid composition within the diets may also affect the cortisol response to stressors, as found in turbot (*Scophthalmus maximus*) fed diets with increased arginine levels and subjected to long-term stressors (Costas et al., 2013). Conversely, dietary supplementation with exogenous tryptophan in Persian sturgeon (*Acipenser persicus*) exposed to acute confinement stress revealed increases in plasma cortisol, while reducing immunosuppression following the stress periods (Hoseini et al., 2016). Thus, variations in the amino acid composition of treatment diets may alter the cortisol response, as bioprocessed ingredients often differ in amino acid profiles due to microbial contributions (Chen, 2013). Future studies evaluating the stress response to these bioprocessed diets may consider equilibrating amino acid profiles or the addition of selected amino acids in excess to evaluate enhancements to the fish stress response.

Glucose levels showed a similar overall pattern, with marked increases in the glucose concentrations of the stressed fish. Similar findings of elevated glucose levels as a secondary stress response have been reported in temperate basses (*M. chrysops, M. saxatilis, M. chrysops x M. saxatilis*), with elevated blood glucose levels present up to 6 hours following an acute stressor (Davis & McEntire, 2009). Within day 60 treatments, the stressed fish showed the lowest blood glucose levels when fed the BSBM diets. It has been well established that blood glucose levels vary with respect to diet composition, such as lipid levels or carbohydrate content, and administration times or periods of

fasting (Barton et al., 1988). The base carbohydrate levels were much greater in the soy ingredients, but dietary differences did not appear to follow previously published results where Atlantic cod (*Gadhus morhua*) fed modified carbohydrate diets experienced a greater secondary stress response than fish fed carbohydrate-free diets (Hemre et al., 1991). Further investigation of carbohydrate composition or lipid profiles (i.e., animal versus plant sources) may elucidate more of the dynamic glucose stress response, in addition to plant-based protein inclusions.

Overall body condition was found to have the greatest K-values in the plant-based diets at the day-60 sampling. There have been some reports of decreased condition factor in hatchery-reared Coho salmon (*Oncorhynchus kisutch*) at higher stocking densities (Fagerlund et al., 1981). It has also been established that low density rearing can also impact variability in weight and length gain in rainbow trout (Kebus et al., 1992). Thus, the observed increase across the plant-based diets may potentially be attributed to dietary treatments, as there was minimal tank density and biomass variation. Also, fish were fed a daily ration based on initial tank biomass, a predicted SGR, and predicted FCR, as stated in the above methodology. Differences in K-values at the final sampling point may indicate some dietary differences that would more closely be examined in a longer term growth study, taking into consideration ingredient palatability and digestibility. Analysis of growth metrics revealed an overall increased performance in the enhanced soy diets, in comparison to the FM control diet. Although these the microbially enhanced diets did not significantly surpass the SBM control in terms of RG and SGR, this variation may be accounted for by some fluctuations in lipid levels during feed production. The SBM and CSPC diets were approximately 3% greater in crude lipid composition than the

microbially enhanced diets, and this differential may account for some of the reduced growth potential.

Hematocrit levels displayed an overall decrease in the stressed fish, and this trend was observed across all diets at both day 30 and day 60. Decreased blood hematocrit levels and hemoglobin concentrations in rainbow trout have been reported to increase with strenuous swimming or acute stress and is related to oxygen transport dynamics (Gallaugher et al., 1995; Biron & Benfey, 1994; Wells & Weber, 1991). Short-term changes in hematocrit levels have also been observed with dietary supplements, such as arginine, inulin, and vitamin c, that have been found to increase blood hematocrit levels in cultured warmwater fish species (Ibrahem et al., 2010; Buentello et al., 2007). The current study demonstrates a decrease in hematocrit with acute stress and deviates from the expected hematocrit increase with stress. The combination of these applied acute stressors (i.e., handling, water volume, and oxygen) or dietary composition may have impacted the ability to increase blood hematocrit during the stress response and warrants further investigation.

Head kidney macrophage RBA showed both increased and decreased superoxide production during the stress events. The CSPC group demonstrated a large difference with the unstressed RBA being dramatically reduced, while the stressed group had the greatest macrophage superoxide production at the end of the trial. It has previously been reported that stressors can impair macrophage function in cultured species (Wedemeyer, 1997). Specifically, adrenergic and corticosteroid stress-related effectors have been found to influence macrophage phagocytosis and decrease cytokine expression levels (Narnaware, 1994; Castro et al., 2011). Conversely, it was found that spleen macrophage

respiratory burst activity increased in rainbow trout subjected to heat shock as a stressor (Wang et al., 2016). It has been reported that there are some direct influences on the hemopoietic tissues (spleen and head kidney) in stressed rainbow trout (Peters, 1985). Further study of cytokine signaling within the tissue during an acute stress event may provide a better understanding of the rainbow trout macrophage dynamics.

Plasma ion analyses showed an overall decrease in chloride and sodium ions within stressed groups, while the potassium ion concentrations showed a general increase. Similar ion fluctuations were trends were noted in Arctic charr (*Salvelinus alpinus*) subjected to temperature stressors and rainbow trout subjected to exhaustive exercise (Lyytikäinen et al., 2002; Kakisawa et al., 1995). The calcium ion fluctuations appeared to be less distinct, with trends of increases and decreases across treatments and within groups. Ion concentration fluctuations have been attributed to the shifts induced by increased muscle activity during the stress event, and this overall ion fluctuation has been previously observed in rainbow trout subjected to handling stressors (Laidley & Leatherland, 1988).

This stress study provides an initial understanding of the potential benefits or physiological enhancements associated with the inclusion of bioprocessed plant-based products in aquafeeds. Although this study focuses on the acute phase stress response, more information on responses to chronic stressors may provide more definitive, long-term rearing information for fish culturists. Thus, more long-term improvements to mitigate stress within aquaculture facilities may be considered, as opposed to stress reduction for short-term events such as net handling or transport. As producers move toward the potential to maximize stocking densities and rearing efficiency, the inclusion

of microbially enhanced plant-based protein sources may translate into greater production yields. In turn, more efficient production facilities will promote the expansion of domestic aquaculture and potentially mitigate trade deficits associated with finfish imports.

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Tables

Table 1. Ingredient proximates of experimental protein sources (as is basis) used in both feeding trials. Dietary treatments were fish meal (FM), defatted soybean meal (SBM), commercial soy protein concentrate (CSPC), and bioprocessed soybean meal (BSBM-L [low inclusion]; -H [high inclusion]).

Proximates	**FM**	**SBM**	**Soycomil-P***	**BSBM**
Crude Protein (g 100g^{-1})	65.54	45.50	65.00*	63.80
Crude Lipid (g 100g^{-1})	8.50	1.71	1.00*	0.56
Crude Fiber (g 100g^{-1})	0.10	5.16	3.50*	7.31
Ash (g 100g^{-1})	18.74	6.02	6.5*	3.80
Moisture (%)	8.01	11.90	7.00	4.93

*Specifications from manufacturer, ADM (Chicago, IL)

Table 2. Experimental diet formulations and analyzed proximate composition of trial diets. Dietary treatments were fish meal (FM), defatted soybean meal (SBM), commercial soy protein concentrate (CSPC), and bioprocessed soybean meal (BSBM-L [low inclusion]; -H [high inclusion]).

Ingredient (g 100 g^{-1} dmb)	FM	SBM	CSPC	BSBM-L	BSBM-H
Menhaden fishmeal[a]	15.00	8.00	12.00	12.00	8.00
Soycomil-P[b]	0.00	0.00	25.00	0.00	0.00
BSBM[c]	0.00	0.00	0.00	25.00	35.54
SBM[d]	0.00	35.00	0.00	0.00	0.00
Wheat midds[e]	18.00	7.96	13.25	13.25	11.26
Whole cleaned wheat[f]	18.00	10.00	17.94	15.60	14.87
Empyreal[g]	5.00	5.00	0.00	0.00	0.00
Poultry meal[h]	14.32	8.00	5.00	5.00	4.00
Feathermeal[h]	7.50	4.00	7.50	7.50	5.00
Blood meal[i]	5.00	3.00	4.98	2.50	2.00
Vitamin premix[j]	1.25	1.25	1.25	1.25	1.25
Mineral premix[k]	1.50	1.35	1.50	1.50	1.75
Stay-C[l]	0.02	0.02	0.02	0.02	0.02
Lysine[m]	2.10	1.80	2.00	2.00	2.00
Methionine[m]	0.75	0.60	0.75	0.75	0.75
Choline[n]	0.06	1.00	0.60	0.60	0.06
Fish oil[o]	11.50	13.02	12.85	12.85	13.50
Totals	100.00	100.00	100.00	100.00	100.00
<u>Proximate Composition (g 100 g^{-1} dmb)</u>					
Crude Protein	44.47	41.14	41.26	41.53	41.87
Crude Lipid	19.50	14.36	14.55	11.11	10.80
Crude Fiber	2.67	3.09	3.61	3.81	3.79
Ash	8.02	7.70	8.22	7.96	7.11
Moisture	5.50	4.60	4.45	4.60	5.53

[a] Special Select, Omega Protein, Houston, TX; [b] Soycomil DM ; [c] Prairie AquaTech, Brookings, SD; [d] South Dakota Soybean Processors, Volga, SD; [e] Consumers Supply Distributing, Sioux City, IA; [f] AgFirst Farmers Cooperative, Brookings, SD; [g] Cargill Corn Milling, Blair, Nebraska; [h] Tyson Foods, Springdale, AR; [i] Mason City By-Products, Mason City, IA; [j] ARS 702 premix, Nelson and Sons, Murray, UT; [k] ARS 640 trace mix, Nelson and Sons, Murray, UT; [l] DSM Nutritional Products, Parsippany, NJ; [m] Pure Bulk, Roseburg, OR; [n] BalChem Corporation, New Hampton, NY; [o] Virginia Prime Gold, Omega Protein, Houston, TX.

Table 3. Tank biomass gain (g), relative growth (RG; %), specific growth rate (SGR; %), and spleen somatic index (SSI) of rainbow trout sampled at day 60. Values shown as mean ± SEM. FM (fishmeal), SBM (soybean meal), BSBM (bioprocessed soybean meal), CSPC (commercial soy protein concentrate), L (low inclusion), H (high inclusion).

	FM	**SBM**	**CSPC**	**BSBM-L**	**BSBM-H**
Tank Biomass Gain (g)	1045±85	1078±79	1244±55	1139±38	1151±69
RG (%)	433±36	476±38	520±32	471±30	441±12
SGR (%)	1.96±0.16	2.17±0.15	2.37±0.12	2.16±0.12	2.04±0.06
SSI	0.12±0.01	0.10±0.01	0.10±0.01	0.12±0.00	0.12±0.01

Table 4. Plasma cortisol (ng mL^{-1}) of trout sampled at days 30 and 60. Plasma cortisol from trout sampled at day 0 was found to be 0.16 ng mL^{-1}. Values shown as mean ± SEM. FM (fishmeal), SBM (soybean meal), BSBM (bioprocessed soybean meal), CSPC (commercial soy protein concentrate), L (low inclusion), H (high inclusion).

	FM	**SBM**	**CSPC**	**BSBM-L**	**BSBM-H**
Day 30 Unstressed	0.21±0.05	0.29±0.13	1.55±1.39	0.16±0.00	0.16±0.00
Day 30 Stressed	8.53±3.44	8.21±1.41	10.82±1.21	12.97±2.32	16.89±5.80
Day 60 Unstressed	0.52±0.36	0.69±0.50	0.16±0.00	0.26±0.10	0.16±0.00
Day 60 Stressed	5.11±2.08	6.33±3.64	13.92±4.27	4.07±1.22	6.68±3.06

Figures

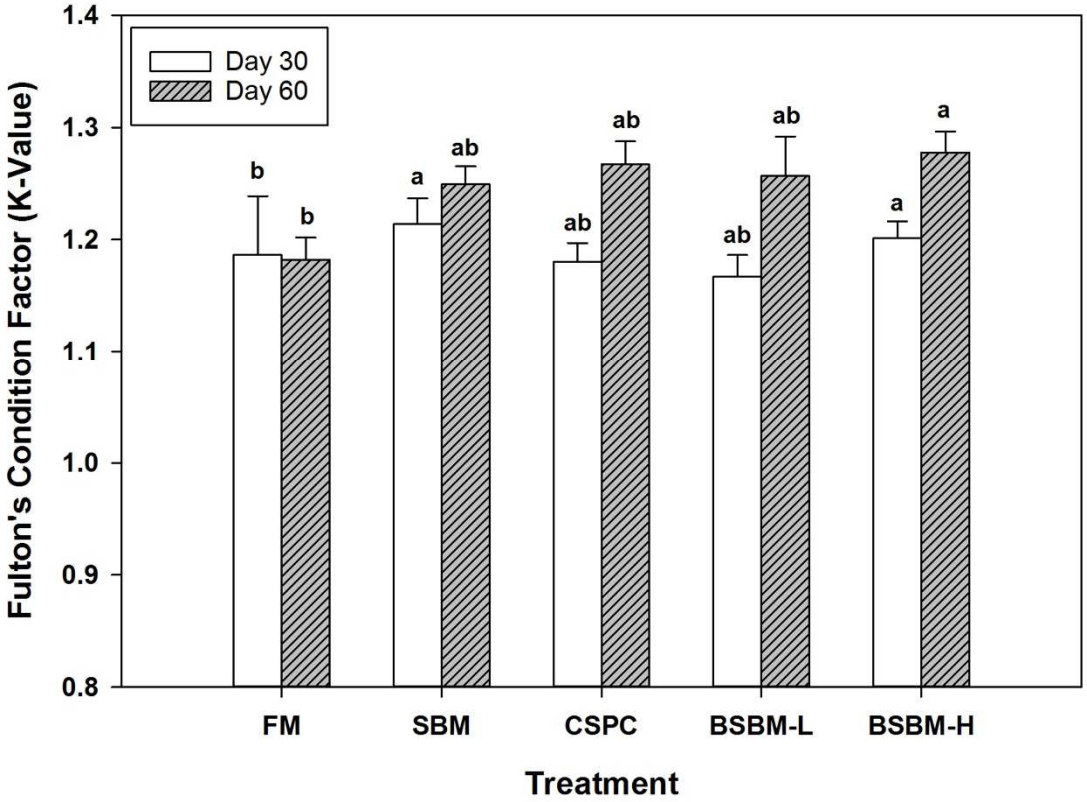

Figure 1. Fulton's condition factor (K-value) of rainbow trout sampled at days 30 and 60. Day 0 condition factor was 1.03±0.01. Different letters denote significant differences between dietary treatments on each sampling date (day 30 and day 60). Dietary treatments were fish meal (FM), defatted soybean meal (SBM), commercial soy protein concentrate (CSPC), and bioprocessed soybean meal (BSBM-L [low inclusion]; -H [high inclusion]).

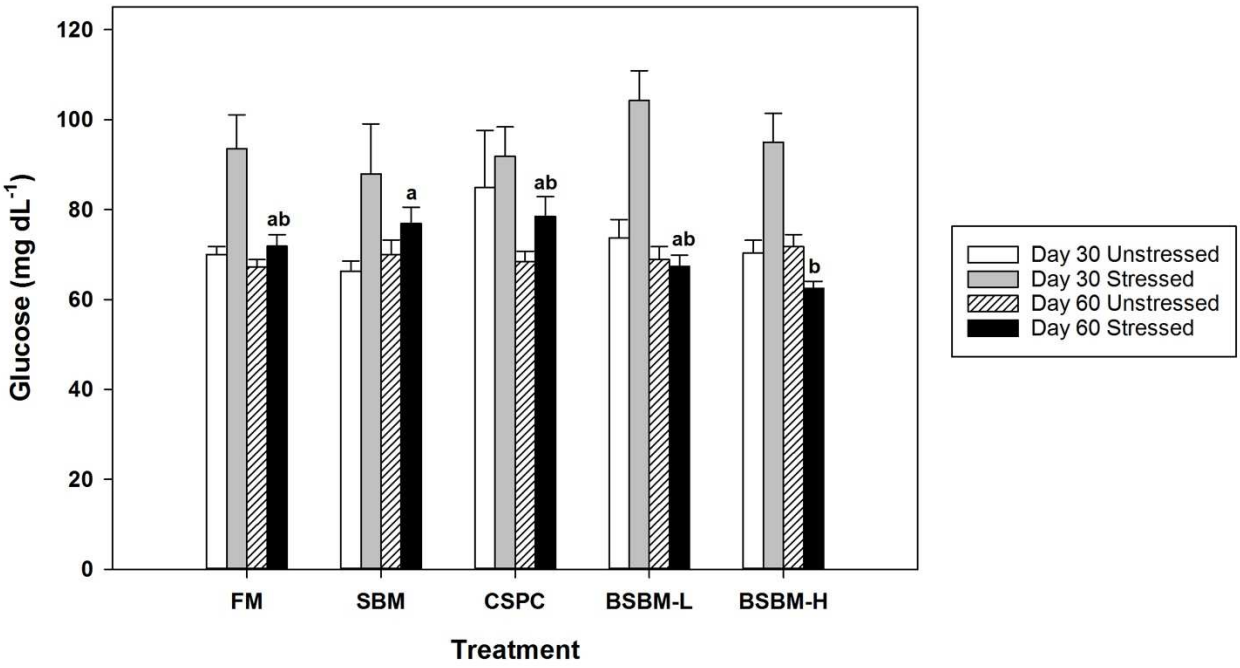

Figure 2. Blood glucose (mg dL^{-1}) of stressed and unstressed rainbow trout sampled at days 30 and 60. Baseline blood glucose from trout sampled at day 0 was 82.07±2.20 mg dL^{-1}. Different letters denote significant differences between dietary treatments for each group at each sampling date. Dietary treatments were fish meal (FM), defatted soybean meal (SBM), commercial soy protein concentrate (CSPC), and bioprocessed soybean meal (BSBM-L [low inclusion]; -H [high inclusion]).

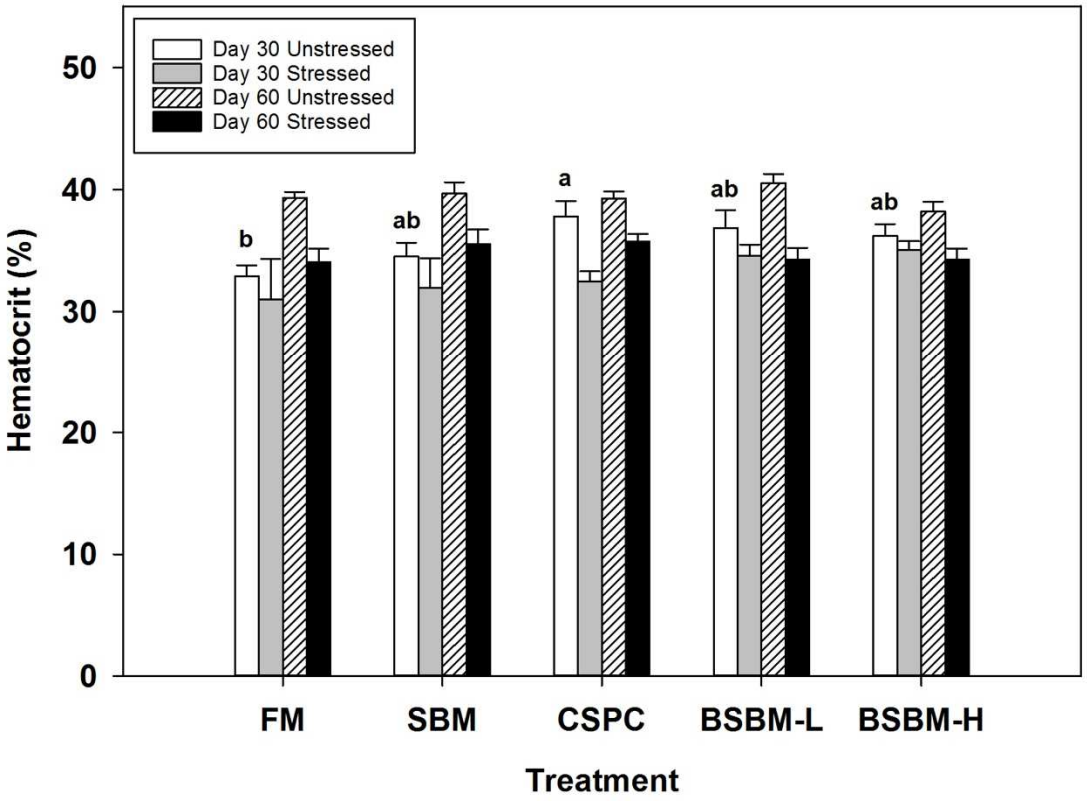

Figure 3. Blood hematocrit (%) of stressed and unstressed rainbow trout sampled at days 30 and 60. Baseline hematocrit from trout sampled at day 0 was 37.17± 1.28 %. Different letters denote significant differences between dietary treatments for each group at each sampling date. Dietary treatments were fish meal (FM), defatted soybean meal (SBM), commercial soy protein concentrate (CSPC), and bioprocessed soybean meal (BSBM-L [low inclusion]; -H [high inclusion]).

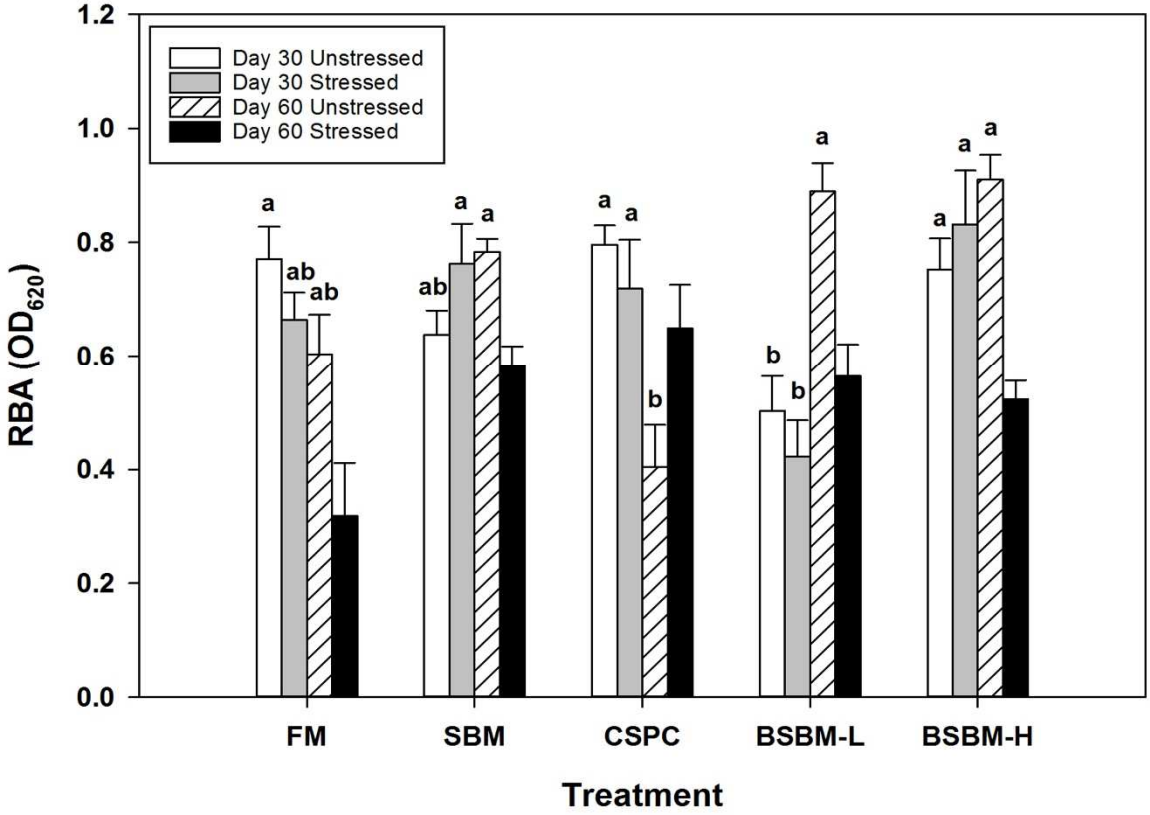

Figure 4. RBA of head kidney macrophages (OD_{620}) of stressed and unstressed rainbow trout sampled at days 30 and 60. Baseline RBA from trout sampled at day 0 was 0.733± 0.030. Different letters denote significant differences between dietary treatments for each group at each sampling date. Dietary treatments were fish meal (FM), defatted soybean meal (SBM), commercial soy protein concentrate (CSPC), and bioprocessed soybean meal (BSBM-L [low inclusion]; -H [high inclusion]).

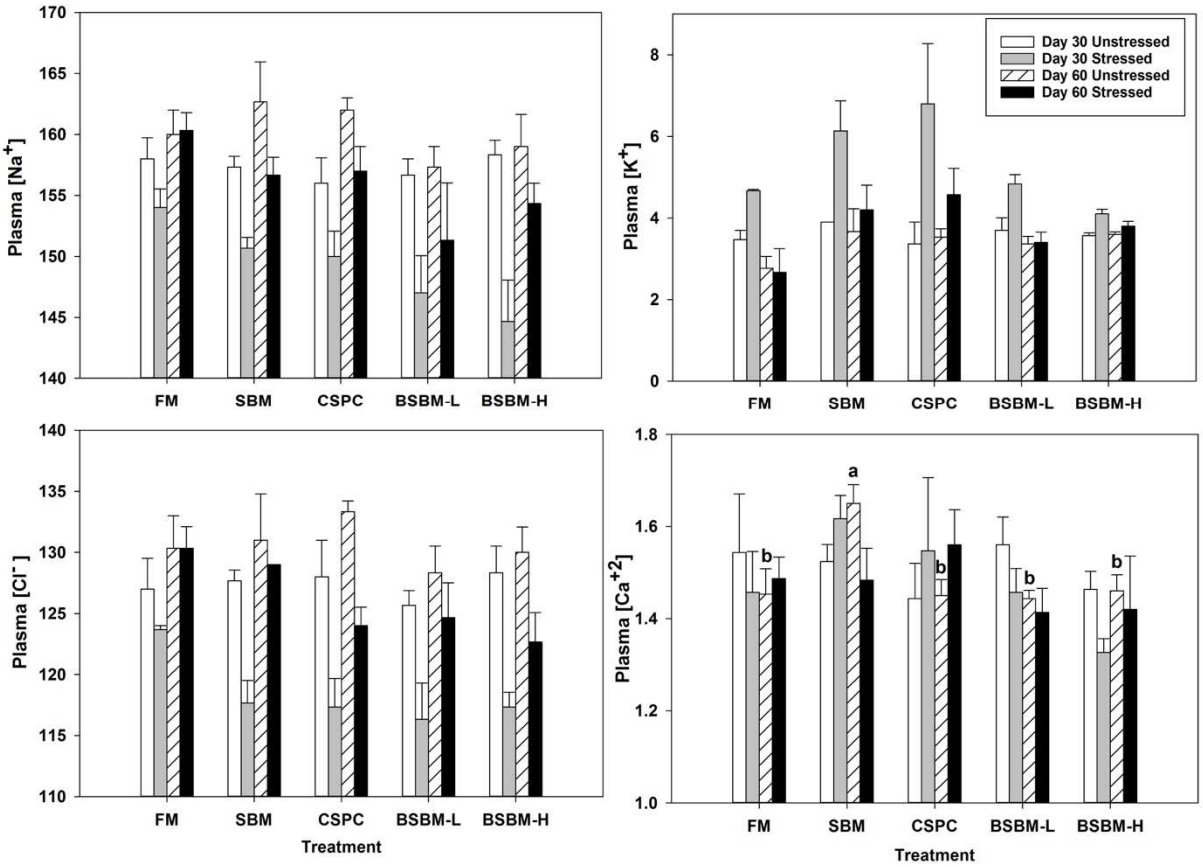

Figure 5. Plasma ions (Na^{+}, K^{+}, Cl^{-}, Ca^{+2}; mmol L^{-1}) of stressed and unstressed rainbow trout sampled at days 30 and 60. Baseline mineral profile for fish sampled at day 0 was Na^{+}=160.6±1.5, K^{+}=3.5±0.2, Cl^{-}=131.6±1.2, Ca^{+2}=1.56±0.02. Different letters denote significant differences between dietary treatments for each group at each sampling date. Dietary treatments were fish meal (FM), defatted soybean meal (SBM), commercial soy protein concentrate (CSPC), and bioprocessed soybean meal (BSBM-L [low inclusion]; -H [high inclusion]).

CHAPTER 7. RESEARCH NEEDS

The research trials performed within this dissertation evaluated changes in fish immunology and physiology following the administration of bioprocessed soy-based (BSBM) aquafeeds. As our domestic aquaculture sector rapidly expands, there is a need to optimize finfish production while ensuring adequate fish health and nutrition. As aquafeeds constitute a large portion of operating costs, sustainable and high-performance BSBM will allow for producers to spend less monetary resources on feed costs and instead focus on potential enhancements to production systems and stocking densities. Further, through the use of these bioprocessed ingredients, producers may also receive the added immunostimulatory benefits for their growing stocks. Thus, lowered feed costs and nutritionally complete BSBM may potentially (and perhaps indirectly) bolster a more developed domestic sector.

All of these feeding trials were designed to provide impactful results and suggestions to be implemented in commercial aquaculture operations. Following the completion of these studies, there remains to be some further research needs and subsequent follow-on trials that will further elucidate dietary impacts on aquaculture immunology and fish health. The advancement of knowledge within this field of research will provide aquaculture industry partners with a considerable amount of knowledge regarding the fish immune response to BSBM-based protein sources, as well as new management practices within these aquaculture facilities may allow for improved production numbers.

To date, there is limited information on immune defense modulations and changes to the gut microbiota in cultured fishes and these studies provide some preliminary insight into the potentially value-added health benefits provided with dietary BSBM inclusions. The aquaculture sector has been investigating diet-based immunostimulants for decades, however, the growing amount of cultured species and interspecies variation with immune responses has necessitated more involved research to elucidate both mechanism and commercial opportunity. Thus, in many cases, fish immunologists have researched "effector" functions or results, while research endeavors involving mechanism-related questions have come to light in order to disseminate results across a many species. The aforementioned trials spanned a great deal of research space, ranging from innate immune studies to acute stress responses.

Thus, the following research needs may potentially be evaluated for continued investigations into fish health with respect to bioprocessed soy-based products:

1. Although EXOP dosing levels for enhanced innate immune attributes were determined in Chapter 2, there are still some aspects of the original research questions that warrant further investigation. In a concurrent growth trial incorporating the commercial EXOP, there were some palatability issues associated with the larger inclusion rates. Thus, larger inclusion levels may not be feasible for producers, as a sacrifice in growth performance is not likely to be forfeited in a commercial setting. Thus, future palatability and formulation optimizations may be required to balance immunostimulation with adequate performance metrics.

The EXOP dosing trial only provided a short-term snapshot of fish fed a constant ration. As some aspects of immunostimulation are closely related to feeding regimen, variation in feeding frequency and/or altering immunostimulants dosing may also elucidate innate immune changes in an effort to optimize dietary inclusion rates. Lastly, if immunostimulants can be more concentrated as dietary supplements, this may allow for minimal inclusions within aquafeed formulations while providing the necessary doses to potentially modulate fish health.

2. Results from the mineral supplement trial indicated some changes to yellow perch innate immune parameters, although there appeared to be some variability within treatments. Some of this variation may be accounted for within the genetic composition of the trial fishes. That is, the yellow perch used in the study came from wild-caught populations and there may be some immunological differences as a result of rearing conditions in a non-intensive setting. Overall, the accompanying rainbow trout trials appeared to have provided less unevenness within dietary treatments. It is well-established that fish are quite sensitive to vitamin and mineral inclusions and a homogenous lot of yellow perch may produce more discernable findings for some immune metrics. Further investigations of these mineral supplements in other commercially-relevant aquaculture species may further elucidate the health-related benefits and potentials for optimized mineral profiles in commercial aquafeeds. These mineral results are also important for aquafeed ingredient

producers, as a favorable, intrinsic mineral profile may negate the need for synthetic vitamin supplementation. Further, in an effort to determine mineral availability within the diet, it may be appropriate to evaluate the inclusions of additional or alternative mineral compounds (i.e. additional forms of zinc and selenium containing compounds) that may elicit different immunostimulatory properties.

3. *F. psychrophilum* dynamics were evaluated in Chapter 4, and provided some novel findings on diet-related modulations on rainbow trout mortality rates and innate immune parameter changes. The strain incorporated in the trial was a recently archived isolate, so the virulence and pathogenicity of the culture was unknown. In a separate follow-on study, relatively larger rainbow trout were intraperitoneally challenged with strain 11730 following 30 days of dietary administration. Trial results indicated a decrease in mortality across dietary treatments, and this is likely accounted for by fish size. This deviation from the aforementioned trial confirms the need to consider fish size and health status during the evaluation of strain virulence.

   Although no major fluctuations in IL-1β and TNF-α concentrations were observed in the spleen and kidney tissue at pre- and post-challenge sampling, further investigation of cytokine profiles may disclose more details on the orchestrated immune response. Further, the determination of bacterial load in spleen may also clarify aspects of strain-specific *F. psychrophilum* pathogenicity.

As research efforts focus on vaccine development for this pathogen, the potential inclusion of these potentially immunostimulatory bioprocessed products may contribute to the creation of an oral vaccine delivery platform. The bioprocessed ingredient may further act in an adjuvant-like manner and provide an enhanced protective antibody development. The combination of hatchery disease management (loss mitigation) and dietary enhancements may provide a new avenue of disease prevention for salmonid producers.

4. The gastrointestinal microbiota work has become of interest to the aquaculture field within the past decades and researchers are discovering the importance of microbiota on fish nutrition and health. Although the trial provided a framework to evaluating BSBM ingredients in rainbow trout diets, many of the microbiota-linked effector mechanisms within the gut remain unknown. There is a wide array of host-pathogen interactions that take place within both the proximal and distal intestine that remained to be discerned through fish metabolism, nutrition, microbiology, and immunology studies. As the gut conditions (pH, enzyme activity, presence of pathogens) influences the species composition of the microflora, further research is needed to better understand which of host-pathogen and host-colonization interactions. Additionally, immunological markers (gene expression, IgT dynamics, and apparent cytokine circulation) within the intestinal tissues would also provide evidence for the effector functions of specific pathogens and the fish health conditions that may allow for beneficial gut bacteria to flourish, from a

molecular level. The ability to determine the effects of the characterized microbiota on digestive enzyme production and gut immunity in fish would provide a foundation for further research regarding the importance of formulating aquafeeds with the intention of selecting for a desired gut microflora diversity.

5. The evaluation of the fish stress response has many pertinent applications for fish producers and provides important management implications for rearing densities. The included trial evaluated the influence of acute stressors in rainbow trout fed BSBM-based aquafeeds. As acute stressors are of great importance for assessing transport and stocking guidelines, the current study did not evaluate long-term stress response physiology. Chronic stressors will exert a greater effect on the fish immune system, as the fish diverts metabolic resources toward survival and respiration dynamics. The incorporation of a long-term chronic stress trial would allow for an evaluation of diets in a setting that would be conducive to domestic aquaculture facilities. In opposition to stressors activating the primary stress response, the long-term trial would also allow for researchers to observe changes to growth performance. Again, the evaluation of a BSBM-based aquafeed may exert some immunostimulation from dietary intake, as well as an enhanced growth response-two factors that may potentially assist with stress management for production stocks. Thus, further investigations regarding the use of BSBM

diets to mitigate stress-related changes in cultured fishes would be a beneficial contribution to aquaculture physiology research.